

IGF-I Is a Mitogen Involved in Differentiation-related Gene Expression in Fetal Rat Brown Adipocytes

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Abstract. Fetal rat brown adipocytes at time zero of culture constitute a population of cells of broad spectrum, as estimated by cell size, endogenous fluorescence and lipid content, and show an intrinsic mitogenic competence. They express constitutively early growth-related genes such as *c-myc*, *c-fos*, and β -actin, tissue specific-genes such as the uncoupling protein (UCP) and the lipogenic marker malic enzyme (ME). Fetal brown adipocytes bear a high expression of insulin-like growth factor receptor (IGF-IR), and show a high affinity IGF-I specific-binding to its receptor, and a high number of binding sites per cell. After cell quiescence, insulin-like growth factor I (IGF-I) was as potent as 10% FCS in inducing DNA synthesis, cell number increase, and the entry of cells into the cell-cycle. In addition, IGF-I or 10% FCS for 48 h increased the percentage of [³H]thymidine-labeled nuclei as compared to quiescent cells. Single cell autoradiographic microphotographs show typical multilocular

fat droplets brown adipocytes, resulting positive to [³H]thymidine-labeled nuclei in response to IGF-I. IGF-I increased mRNA expression of the early-response genes *c-fos* (30 min), *c-myc* (2 and 24 h), and *H-ras* (4 and 24 h). 10% FCS also increased *c-fos* and *c-myc*, but failed to increase *H-ras* as an early event. IGF-I or 10% FCS, however, similarly increased the mRNA late expression of *c-myc*, *H-ras*, *c-raf*, β -actin, and glucose 6-phosphate dehydrogenase (G6PD) at 72 h, as compared to quiescent cells. IGF-I or FCS maintained at 24 h or increased at 48 and 72 h UCP mRNA expression. The results demonstrate that IGF-I is a mitogen for fetal rat brown adipocytes, capable of inducing the expression of early and late growth-regulated genes, and of increasing the lipogenic marker ME and the tissue-specific gene UCP, suggesting the involvement of IGF-I in the differentiation as well as in the proliferation processes.

INSULIN-like growth factor I (IGF-I)¹ is an extracellular factor involved in differentiation and/or proliferation of eukaryotic cells (12, 14). Several lines of investigation suggest that both processes share common IGF-I signal transduction pathways, in which p21 Ras is a crucial intermediate.

With regard to the differentiation process, insulin or IGF-I is involved in the meiotic maturation of *Xenopus* oocyte. This process was inhibited when Ras antibody Y13-259 was microinjected into the cells in the presence of insulin, suggesting a direct role of the Ras proteins in the insulin/IGF-I signaling transduction pathways involved in triggering that process in these amphibian cells (13, 23). Differentiation of mammalian 3T3 L1 cells into adipocytes shows an absolute requirement for insulin or IGF-I (39). However, the expression of transfected Ras oncogenes in 3T3 L1 leads to differen-

tiation into adipocytes in the absence of externally added insulin or IGF-I (4). Moreover, transfected dominant inhibitory Ras mutants specifically block the differentiation process triggered by insulin, suggesting that endogenous ras proteins are mediators of insulin signaling in these cells, while other oncogenes, such as *src* or *trk*, could not induce such a differentiation process (4, 35).

With regard to the proliferation processes, IGF-I (nM) or insulin (μ M) are very poor mitogens in mouse fibroblast cell lines (14). In this system, insulin is unable to induce the expression of *c-fos*, *c-jun*, and p33 (8). Overexpression of insulin receptors or p21 *H-ras* produced mitogenesis in NIH 3T3 transfected cells. In those cells insulin stimulation produced an increase in Ras GTP/Ras GDP levels, while PDGF or EGF stimulation of cells expressing high levels of the cognate receptors did not increase p21 Ras GTP levels (8). Quiescent BALB/c 3T3 cells enter the S phase of the cell cycle when growth factors are provided in a specific order, Ras function being essential for the ability of IGF-I to stimulate entry into S phase from late G1 (progression), but not for the ability of PDGF or EGF to induce *c-fos* mRNA early expression in the G0/G1 transition (initiation) (26, 27). In addition, overexpression of IGF-I receptors (IGF-IR) in BALB/c 3T3

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1. *Abbreviations used in this paper:* G6PD, glucose 6-phosphate dehydrogenase; IGF-I, insulin-like growth factor I; IGF-IR, insulin-like growth factor receptor; ME, malic enzyme; UCP, uncoupling protein.

cells produced mitogenesis and the induction of *cdc2* mRNA (a late growth-regulated gene) in the presence of IGF-I, but in the absence of PDGF (42), and in NIH 3T3 cells a ligand-dependent transformation (22). In non-fibroblastic mammalian cells, however, although not yet described in adipocytes, IGF-I caused a transient increase of *c-fos* without changes in *c-myc* in mouse osteoblasts (29), and in canine kidney cell lines (18), an increase in *c-fos* and *c-myc* in murine embryonic and tumor cells (32), and an increase in *c-fos* and *c-jun* in primary cultures of rat oligodendroglial cells (5), related to the stimulation of DNA synthesis and proliferation.

Previous findings show that fetal brown adipocytes in primary culture increase DNA synthesis and cell number, and DNA, RNA, and protein contents in response to IGF-I (44). In these cells, IGF-I induces the genetic expression of glucose 6-phosphate dehydrogenase (G6PD), an enzyme involved in providing ribose-5-phosphate for DNA synthesis in growing cells (44). The aim of the present work is to investigate, first, the expression of proliferation-related and differentiation-related genes in quiescent primary fetal brown adipocytes stimulated in response to IGF-I, and, second, the relationship between IGF-I-stimulated proliferation and IGF-I-stimulated differentiation. Our results show that IGF-I is a mitogen per se that increases the expression of early and late genes involved in fetal rat brown adipocytes proliferation, and also increases the expression of the lipogenic marker malic enzyme (ME) and the tissue-specific gene uncoupling protein (UCP). In addition, differentiated fetal brown adipocytes, showing their typical multilocular fat droplets distribution, become positive for [³H]thymidine-labeled nuclei in response to IGF-I.

Materials and Methods

Cell Culture

Brown adipocytes were obtained from interscapular brown adipose tissue of 20-d fetuses of Wistar rats and isolated by collagenase dispersion as described (25). Cells were plated at 10⁶ cells/60-mm tissue culture plates in 2.5 ml of MEM supplemented with 10% FCS (Imperial Laboratories, Hampshire, U.K), the presence of serum being essential to allow cell attachment to the plastic surface of the plates. After 4–6 h of culture at 37°C, cells were rinsed twice with PBS and 70% of the initial cells were attached forming a monolayer that under inverse light microscopy represented ~30% of confluence (*time 0 of culture*). At this time, the study of cell cycle by propidium iodine staining followed by flow-cytometric analysis revealed that 15% of cells are in S+G₂+M phases of the cell cycle and 85% of cells are G₀/G₁ (Fig. 1 E). Cells were maintained for 20 h in a serum-free medium supplemented with 0.2% (wt/vol) BSA to assure inhibition of the intrinsic mitogenic competence of fetal cells. At this time, the study of cell cycle revealed that >95% of cells are in G₀/G₁ phases of the cell cycle. This time (20 h serum-starved) is the starting point for mitogenic stimulation. Quiescent cells were further cultured for 30 min, 2, 4, 6, 8, 24, 48, and 72 h in the presence of either IGF-I (1.4 nM, except in concentration-dependent experiments) (Boehringer, Mannheim, Germany), or 10% FCS as positive control for cell proliferation, or in the absence of serum and growth factors as control for cellular quiescence.

Determination of [³H]Thymidine Incorporation into Acid-Insoluble Material

DNA synthesis was determined after 24, 48, and 72 h of cell culture in the presence or absence of IGF-I, or 10% FCS, by [³H]thymidine incorporation (0.2 μCi/ml) (Amersham, Buckinghamshire, UK) into acid-insoluble material over the last 4 h of culture. Determination of radioactivity in TCA-

insoluble material was as described (30). Results are expressed as dpm/dish or as percentage of radioactivity incorporated by untreated cells (100).

Nuclear Labeling and Autoradiography

For nuclear labeling, quiescent cells (20 h serum-starved) were cultured for 48 h in the presence or absence of IGF-I, or 10% FCS, and [³H]thymidine (2 μCi/ml; 1 μM). At the end of the incubation, cultures were rinsed twice with PBS, extracted with TCA and washed with ethanol. The dishes were coated with Kodak AR 10 stripping film, exposed for 2 wk, developed, and photographed. The nuclei were lightly stained with Giemsa stain and the number of labeled and unlabeled cells of several microscopic fields were counted, and the results expressed as described (38).

Flow Cytometric Analysis

Several cellular parameters were determined by flow cytometric analysis of individual cells, such as size, endogenous fluorescence, cytoplasmic lipid content by Nile red fluorescence, cell number, and analysis of cell cycle. Cells were detached from plates by addition of 0.05% trypsin-0.02% EDTA. After 2–3 min, trypsinization was stopped with 10% FCS in the culture medium. All measurements were performed in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Excitation light was 488 nm. Size signals (FSC-H) were measured through a filter BP 488/10 nm. Endogenous flavin green fluorescence (FL1-H) was measured through a filter BP 530/30 nm (43). Cytoplasmic lipid content was determined by Nile red fluorescence emission 530 (BP 530/30 nm) (16). Cell number was determined in the flow cytometer. The percentages of cells in G₀/G₁ and in S+G₂+M phases of the cell cycle were determined after staining nuclei with propidium iodine by using the Cycle test DNA reagent kit (Becton-Dickinson), measured in a Double Discriminator Module and computer analyzed.

(¹²⁵I)IGF-I Binding

Quiescent monolayers (20 h serum-free) were incubated for 3 h at 20°C with 0.03 nM (¹²⁵I)IGF-I (80 μCi/μg) in binding buffer 25 mM Hepes-PBS containing 1 mg/ml BSA in the absence or presence of graded concentrations of unlabeled IGF-I. Triplicate dishes were used for all data point. At the end of the incubation, monolayers were rinsed three times with PBS-BSA and solubilized in 0.1 N NaOH-1% SDS-2% Na₂CO₃. Radioactivity was counted in a γ-Packard counter. Nonspecific binding was defined as radioactivity that remained bound in the presence of a 1,000-fold excess of unlabeled IGF-I. Scatchard plots and binding sites were calculated from three separate experiments, as described (33, 34).

RNA Extraction and Analysis

At the end of the culture time, cells were washed with ice-cold PBS and the monolayer was lysed directly with RNazol B (Biotecx Lab, Dallas, TX) after the protocol supplied by the manufacturer for total RNA isolation (10). For brown adipocyte primary cultures the yield was 7–23 μg of RNA per 60-mm tissue culture plates. Poly(A)⁺ RNA was isolated by affinity chromatography on oligo(dT)cellulose columns (17). Oligo(dT)cellulose (Collaborative Research, Bedford, MA) was hydrated in DEPC-treated water, placed in a 10-ml polypropylene column (Econo-column, Bio-Rad Labs., Richmond, CA) and equilibrated in binding buffer (0.5 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.5% SDS). Approximately 1 mg of total RNA suspended in 1 ml of binding buffer was heat denatured, applied to the column and washed with 10 ml of binding buffer. Poly(A)⁺ RNA was then eluted by addition of elution buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.05% SDS) and collected in 0.5-ml fractions. The fractions containing Poly(A)⁺ RNA were then pooled and the mRNA was recovered by ethanol precipitation.

Total cellular RNA (10–20 μg) and Poly(A)⁺ RNA (5–10 μg) were submitted to Northern blot analysis, being electrophoresed on 0.9% agarose gels containing 0.66 M formaldehyde, transferred to GeneScreen™ membranes (New England Nuclear Research Products, Boston, MA) using a VacuGene blotting apparatus (LKB, Pharmacia, Uppsala, Sweden) and cross-linked to the membranes by UV light. Hybridization was in 0.25 M NaHPO₄ pH 7.2, 0.25 M NaCl, 100 μg/ml denatured salmon sperm DNA, 7% SDS and 50% deionized formamide, containing denatured ³²P-labeled cDNA (10⁶ cpm/ml) for 40 h at 42°C as described (3). cDNA-labeling was carried out with α³²P-dCTP to a specific activity of 10⁹ cpm/μg of DNA by using a multiprimer DNA-labeling system kit (Amersham, Buckingham-

shire, UK). For serial hybridization with different probes, the blots were stripped and rehybridized sequentially as needed in each case. Probes utilized included v-src (Oncor, Gaithersburg, MD) (41), v-fos (Oncor, Gaithersburg, MD) (46), c-myc (Amer. Type Culture Collection, Rockville, MD) (2), v-raf (Oncor, Gaithersburg, MD) (6), H-ras (kindly provided by E. Santos, Bethesda, MD) (24), β -actin (Oncor, Gaithersburg, MD) (11), UCP (7), ME (28), G6PD (21), IGF-IR (kindly provided by E. Hernandez, Baltimore, MD) (20). The resulting membranes were subject to autoradiography for 1–3 d. Relative densities of the hybridization signals were determined by densitometric scanning of the autoradiograms in a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

Results

Brown Adipocytes at Time 0 of Culture Are a Population of Cells of Broad Spectrum That Expressed Proliferation as Well as Differentiation Markers

Brown adipocytes at time 0 of culture (as described in Materials and Methods) were characterized by flow cytometry for size, endogenous fluorescence, cytoplasmic lipid content by Nile Red fluorescence, and cell cycle (Fig. 1). Cell size signals (FSC) gives a broad peak, indicating a popula-

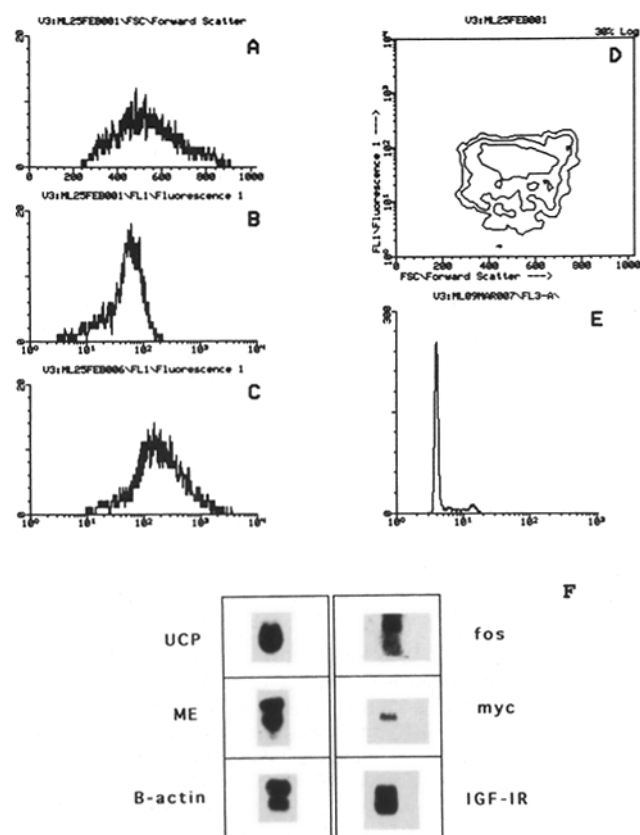


Figure 1. Characterization of fetal brown adipocytes at time 0 of culture. Fetal brown adipocytes at time 0 of culture were characterized by flow cytometry for size (FSC) (A), endogenous fluorescence (FL1) (B), cytoplasmic lipid content by Nile Red fluorescence (FL1) (C), and endogenous fluorescence vs size (D). Cell cycle analysis by propidium iodine staining of nuclei and flow cytometric analysis is shown in E. Poly(A)⁺ RNA (10 μ g) from brown adipocytes before cell culture was submitted to Northern blot analysis and hybridized with various labeled probes F. Representative autoradiograms are shown.

tion of cells with a broad spectrum of size (Fig. 1 A). Endogenous flavin green fluorescence (FL1) gives evidence of the metabolic steady state of living cells. Since brown adipocytes have a high number of mitochondria, the detection of this green fluorescence is possible when flavins are in the oxidized state (43). The analysis of endogenous fluorescence gives a fairly sharp peak with a pronounced tail of low intensity, suggesting at least two populations of cells of high and low metabolic activities, respectively (Fig. 1 B), both with a broad size distribution, as indicated when endogenous fluorescence is represented versus size (Fig. 1 D). Nile Red fluorescence serves as a sensitive detector of cytoplasmic lipid droplets (16). Brown adipocytes, bearing multilocular fat droplets, analyzed for Nile Red fluorescence show a broad peak, indicating again a population of cells of broad spectrum, with a high cytoplasmic lipid content (Fig. 1 C). Thus, the estimation of cell size, endogenous fluorescence, and lipid content indicate that brown adipocytes before culture behave as a population of cells of broad spectrum.

Cell cycle analysis of brown adipocytes at time 0 of culture (Fig. 1 E) indicates that 85% of cell are in G₀/G₁ phase of cycle and 15% of cell are in S+G₂+M. So, since brown adipocytes before cell culture have a high amount of lipid content as well as an intrinsic mitogenic competence, we studied the expression of several genes related to differentiation and proliferation processes (Fig. 1 F). Thus, we checked in poly(A)⁺ RNA by Northern blot analysis the expression of the UCP mRNA, a specific gene of brown adipose tissue highly regulated during development (36), as well as ME, a lipogenic gene highly expressed in brown adipocytes (45). Brown adipocytes before culture expressed constitutively early response genes of cell proliferation such as c-fos, c-myc, and β -actin mRNA (15). A high expression of IGF-IR mRNA (a 7–7.5-Kb isoform) was also found in brown adipocytes.

Brown Adipocytes Bind IGF-I with High Affinity and Expressed a High Number of IGF-I-binding Sites Per Cell

Quiescent brown adipocytes (20 h serum-starved) (as described in Materials and Methods) bind IGF-I with high affinity ($K_d = 4.8$ nM) as shown in the Scatchard plot (Fig. 2 A). A high number of IGF-I binding sites (190,000 sites/cell) was calculated from this plot. Growth of brown adipocytes (determined by [³H]thymidine incorporation into acid-insoluble material) was maximally stimulated by IGF-I at 1.4 nM, as observed in the dose-response curve depicted in Fig. 2 B. This concentration was used in further experiments described below.

IGF-I Increased the Percentage of Cells in S, G₂, and M Phases of the Cell Cycle, [³H]Thymidine Incorporation into Acid-Insoluble Material and Cell Number in Fetal Primary Brown Adipocytes at 72 h

Brown adipocytes showed an intrinsic mitogenic competence at time 0 of culture (15% of cells in S+G₂+M), as above-described from cell cycle analysis by flow cytometry (Fig. 1 E). The maintenance of cells for 20 h in a serum-free medium, as described in Materials and Methods, produced an inhibition of this intrinsic mitogenic competence (<5% of cells in S+G₂+M). This time is the starting point for

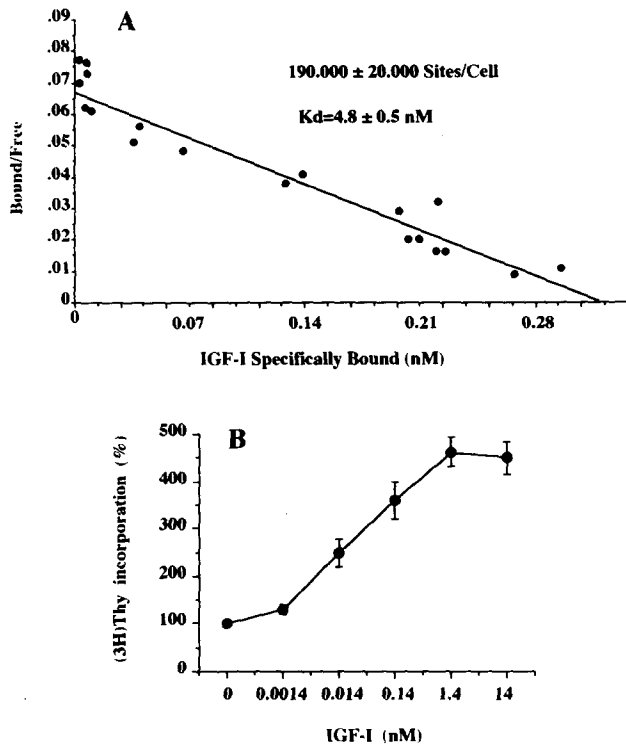


Figure 2. Scatchard plot of IGF-I-binding sites (*A*) and dose-response IGF-I effect on DNA-synthesis (*B*) for fetal brown adipocytes. (*A*) Quiescent cells (20 h serum-starved) were incubated for 3 h with (^{125}I)IGF-I in the absence or presence of graded concentrations of unlabeled IGF-I for receptor binding analysis, as described in Materials and Methods. A representative experiment is shown. K_d and binding sites are means \pm SEM from three independent experiments. (*B*) Quiescent cells (20 h serum-starved) were cultured for 72 h in the presence of graded concentrations of IGF-I for [3H]thymidine incorporation into acid-insoluble material during the last 4 h of culture as described. Results are means \pm SEM ($n = 4-6$) and are expressed as percentage of the radioactivity incorporated by untreated cells (100).

mitogenic stimulation. Cell-cycle analysis after 24, 48, and 72 h in the presence of IGF-I, or 10% FCS, or in untreated cells, is shown in Fig. 3 (*lower panel*). The percentage of fetal brown adipocytes in S+G₂+M was very low (4%), when cells were cultured in the absence of serum and growth factors for 48 or 72 h, as control of cellular quiescence. IGF-I had a low effect on S+G₂+M when present for either 24 or 48 h in the culture medium, respectively. However, after 72 h in the presence of IGF-I, a threefold increase in the percentage of cells in S+G₂+M phases of the cell-cycle occurred, as compared to untreated quiescent cells. The positive control of cellular proliferation showed that the culture of brown adipocytes with 10% FCS produced a low effect at 24, and a threefold increase at 48 and 72 h in the percentage of cells in S+G₂+M phases of the cell-cycle, respectively, as compared to untreated cells.

[3H]Thymidine incorporation into acid-insoluble material in fetal brown adipocyte primary cultures at 24, 48, and 72 h after the addition of either IGF-I, or 10% FCS used as a positive control of cell proliferation, or in the absence of serum and growth factors as negative control of cell prolifer-

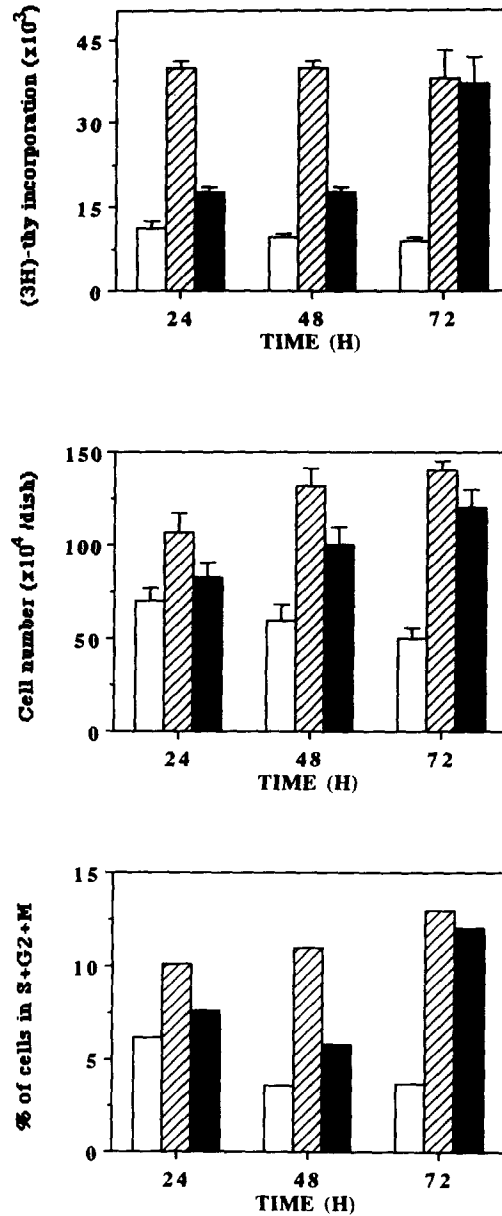


Figure 3. IGF-I increased [3H]thymidine incorporation into acid-insoluble material, cell number and percentage of fetal brown adipocytes in cell cycle at 72 h. Quiescent cells (20 h serum-starved) were cultured for 24, 48, and 72 h in the presence of IGF-I (1.4 nM) (■), or 10% FCS as positive control for cell proliferation (▨), or in the absence of serum and growth factors as control for cellular quiescence (□). (*Upper panel*) [3H]Thymidine incorporation into acid-insoluble material was measured in the last 4 h of culture. Results are mean \pm SEM ($n = 8-10$) and are expressed as dpm/dish. (*Central panel*) Cell number was determined by flow cytometry at the indicated times of culture. Results are means \pm SEM ($n = 4-6$). (*Lower panel*) Cell-cycle study was performed after propidium iodine staining of nuclei and flow cytometric analysis. Results are expressed as percentage of cells in S+G₂+M phases of the cell cycle.

ation (quiescent cells) is shown in Fig. 3 (*upper panel*). IGF-I slightly increased DNA synthesis at 24 and 48 h of treatment. However, at 72 h, IGF-I increased fourfold [3H]thymidine incorporation as compared to quiescent cells. The

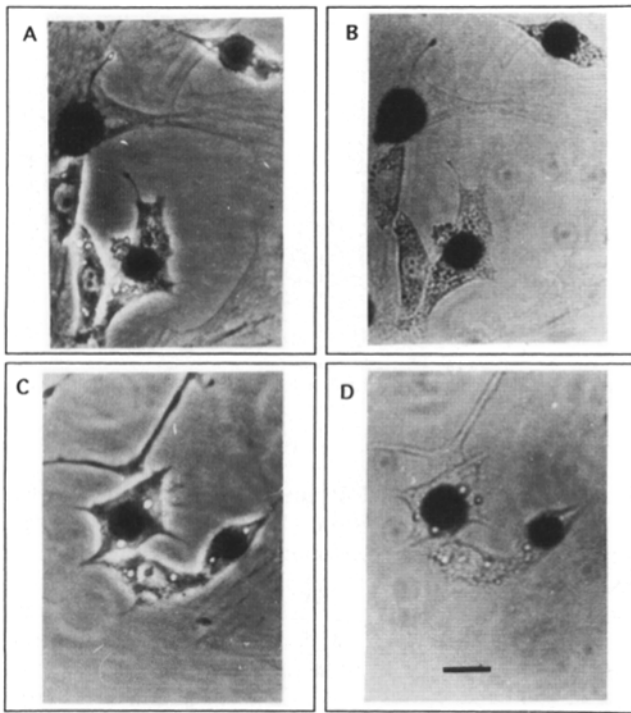


Figure 4. IGF-I induced nuclear labeling in fetal brown adipocytes. Quiescent cells (20 h serum-starved) were cultured for 48 h in the presence of IGF-I and [³H]thymidine, as described in Materials and Methods. The dishes were coated with Kodak AR 10 stripping film, exposed for 2 wk, and developed. (A and C) Phase-contrast microphotographs. (B and D) Light-field microphotographs. Bar, 20 μ m.

presence of 10% FCS produced a fourfold increase in [³H]thymidine incorporation at 24, 48, and 72 h of treatment, compared to untreated quiescent cells.

The effect of IGF-I, or 10% FCS, or cells maintained untreated, on the cell number determined by flow cytometry under the same experimental conditions seen above is shown in Fig. 3 (*central panel*). IGF-I gradually increased cell number throughout the time of culture, producing a twofold increase after 72 h of culture compared to untreated cells. 10% FCS produced a similar effect at 48 h.

Autoradiography of [³H]Thymidine-Labeled Nuclei Demonstrates That IGF-I Produced DNA Synthesis in Single Brown Adipocytes

Besides incorporation of [³H]thymidine into acid-insoluble material for determination of DNA synthesis, the proportion of cells actually synthesizing DNA can be measured by autoradiographic techniques (38). Quiescent brown adipocytes (20 h serum-starved) were incubated for 48 h with [³H]thymidine in the absence or in the presence of IGF-I, or 10% FCS, for determination of the percentage of labeled nuclei after autoradiography, as described in Materials and Methods. In untreated cells, a 13.6 ± 0.7 percent of nuclei were unlabeled, while the presence of IGF-I increased the percentage of labeled nuclei to 45.9 ± 0.8 , and the positive control of cell growth (10% FCS) showed a 48.5 ± 2.0 percent of the labeled nuclei. Microphotographs of several autoradiographic fields of brown adipocytes labeled nuclei cul-

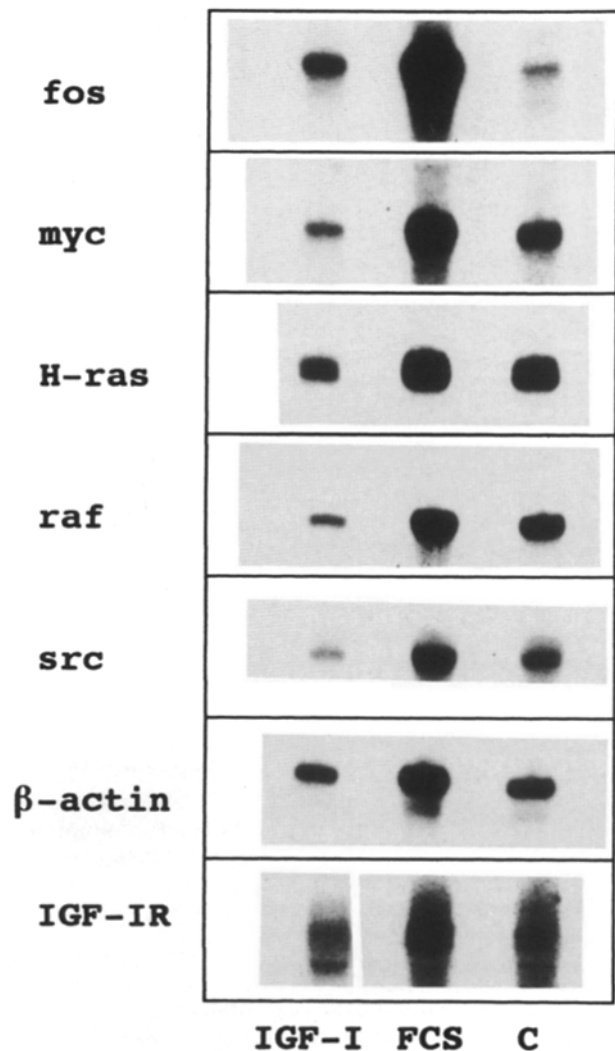


Figure 5. IGF-I induced the expression of *c-fos* mRNA after 30 min of stimulation in fetal brown adipocytes. Quiescent cells (20 h serum-starved) were stimulated for 30 min with IGF-I (1.4 nM), or 10% FCS, as compared to untreated cells (C). Poly(A)⁺ RNA (5–10 μ g) was submitted to Northern blot analysis and hybridized with various labeled probes. Representative autoradiograms are shown.

tured for 48 h in the presence of IGF-I are shown in Fig. 4. These pictures illustrate that single fetal rat brown adipocytes, showing their typical multilocular fat droplets distribution, present positive and negative [³H]thymidine labeled nuclei in the presence of IGF-I.

IGF-I Induced the Expression of c-fos mRNA after 30 min of Stimulation in Fetal Primary Brown Adipocytes

Quiescent fetal brown adipocytes in culture (20 h serum-starved cells) were stimulated for 30 min with IGF-I, or 10% FCS, or maintained untreated. Poly(A)⁺ RNA expression was analyzed by Northern blot as depicted in Fig. 5. After normalization of Poly(A)⁺ RNA loaded by the amount of IGF-IR mRNA detected, as indicated below in the description of Fig. 7, blot densitometric scanning results were expressed as mRNA-fold increase compared to the expression

Table I. Time Course of Gene Induction by IGF-I and 10% FCS: Densitometric Analysis

time (h)	0.5		2		4		6		8		24		72	
	I	F	I	F	I	F	I	F	I	F	I	F	I	F
fos	10	25	-	-	-	-	-	-	-	-	-	-	1.3	3
myc	1.1	1.3	3.5	3	3	1.3	2	2.1	1.1	1.1	2.2	1.8	3	3
H-ras	1	1	1.4	1	3	0.8	1.8	1.3	1.3	1	3	1.9	6	4.2
raf	0.6	0.7	-	-	-	-	-	-	-	-	-	-	23	28
src	1.2	0.9	-	-	-	-	-	-	-	-	-	-	1	1
G6PD	-	-	1	1	1.6	0.5	1.4	0.7	1	0.5	4	1.2	20	10
β -actin	2	1.6	2.1	2	1.8	1	1.4	1.3	1	1.2	2	2.3	2.7	2.4

Autoradiograms shown in Figs. 5 and 7 (from Poly A⁺ RNA) were submitted to densitometry and normalized with the amount of IGF-IR mRNA detected. Autoradiograms from Fig. 6 (total RNA) were submitted to densitometry and normalized with the amount of 18S rRNA visualized by ethidium bromide staining of blots. mRNA content induced by IGF-I or 10% FCS is expressed as fold increase relative to the expression detected in the untreated cell (1 arbitrary unit) at the various times of culture.

detected in the control untreated cells (1 arbitrary unit) (Table I). The presence of IGF-I for 30 min produced a tenfold increase in fos mRNA expression, the expression of c-myc, H-ras, c-raf, c-src, and β -actin mRNA remaining essentially unmodified. Concurrently, the presence of 10% FCS for 30 min produced a 25-fold increase in fos mRNA expression, without changes in the mRNA expression of the other genes analyzed.

IGF-I Induced the Expression of c-myc, β -actin, H-ras, and G6PD mRNA throughout 24 h

A 24 h time-course of IGF-I or 10% FCS effect on mRNA expression of c-myc, β -actin, H-ras, and G6PD was carried out in fetal primary brown adipocytes as compared to untreated quiescent cells. Equal amounts of total RNA were submitted to Northern blot analysis, as shown in Fig. 6. Northern blots were quantified by densitometric scanning analysis and normalized with the amount of 18S rRNA visualized by ethidium bromide staining of blots (Table I). IGF-I increased the expression of c-myc mRNA at 2 h as compared to control cells (3.5-fold). After 2 h, however, the expression of c-myc progressively decreased, reaching at 8 h its lowest content found in the presence of IGF-I. After 24 h, a second peak of c-myc mRNA expression (2.2-fold) was found in the presence of IGF-I. The expression of β -actin mRNA by IGF-I showed a similar pattern throughout the time studied. IGF-I increased H-ras mRNA expression at 4 and 24 h by threefold compared to the untreated cells. Conversely, IGF-I increased G6PD at 24 h as compared to control cells (Fig. 6, Table I). The presence of 10% FCS produced similar results regarding the expression of c-myc and β -actin in fetal brown adipocytes primary cultures, as compared to control quiescent cells. However, 10% FCS failed to increase the expression of H-ras mRNA at 4 and 24 h and the G6PD at 24 h, respectively (Fig. 6, Table I).

IGF-I Increased the Expression of H-ras, c-myc, β -actin, c-raf, and G6PD mRNA at 72 h

Fetal brown adipocytes were cultured for 72 h in the presence of IGF-I, or 10% FCS, or maintained untreated, and poly(A)⁺ RNA was submitted to Northern blot analysis and serial hybridization for several genes studied, respectively (Fig. 7). Since most of these genes are subject to changes in their expression levels related to proliferation and/or differentiation processes undergone in culture, Northern blots

were normalized by the IGF-IR mRNA content found (Table I). In fact, although the expression of IGF-IR mRNA is highly regulated during development in the rat in a tissue-specific manner (48), and though its promoter has a number of features suggesting potential regulatory elements (47), the expression of IGF-IR mRNA remained unchanged in rat granulosa cells after 72 h in the presence of nM concentrations of IGF-I (Hernandez, E., personal communication). Thus, IGF-I for 72 h increased threefold the expression of c-myc and β -actin mRNA, sixfold the expression of H-ras mRNA, 23-fold the expression of raf mRNA and 20-fold the expression of G6PD mRNA in fetal brown adipocytes in culture, as compared to quiescent untreated cells (Fig. 7, Table

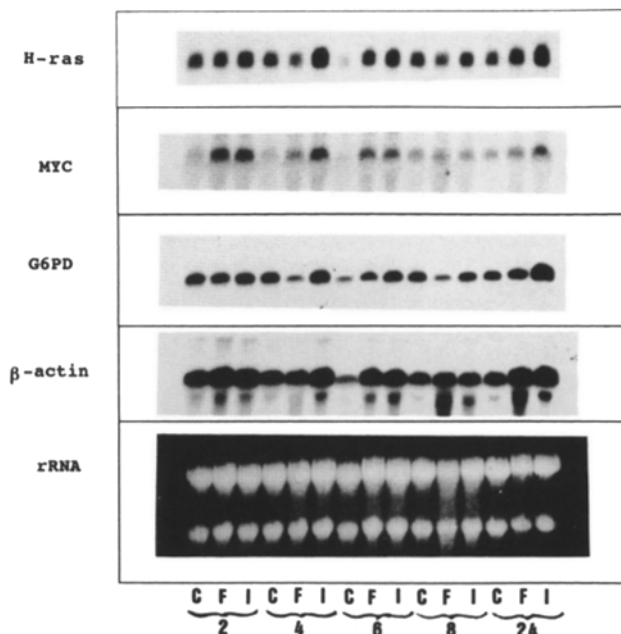


Figure 6. IGF-I induced the expression of H-ras, c-myc, β -actin, and G6PD mRNA. Quiescent cells (20 h serum-starved) were cultured for 2, 4, 6, 8, and 24 h in the presence of IGF-I (1.4 nM) (I), or 10% FCS (F), as compared to untreated cells (C). Total RNA (10 μ g) was submitted to Northern blot analysis and hybridized with various labeled probes. Representative autoradiograms are shown as well as a photograph of Ethidium bromide staining of blots.

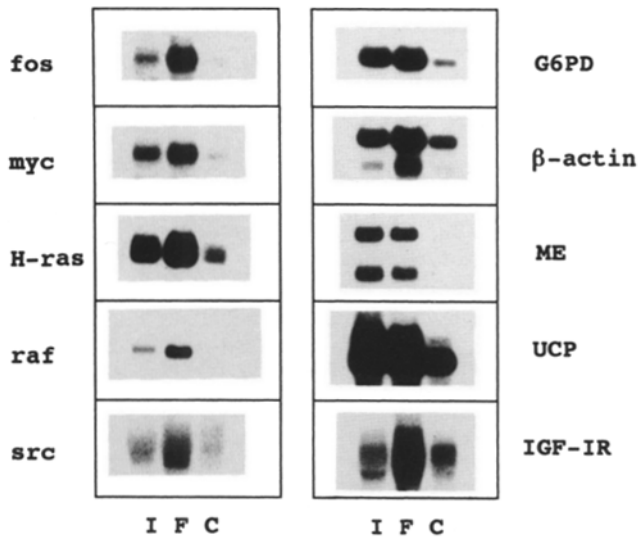


Figure 7. IGF-I increased the expression of H-ras, c-myc, β -actin, c-raf, and G6PD mRNA at 72 h. Quiescent cells (20 h serum-starved) were cultured for 72 h with IGF-I (1.4 nM) (I), or 10% FCS (F), as compared to untreated cells (C). Poly(A)⁺ RNA (5–10 μ g) was submitted to Northern blot analysis and hybridized with various labeled probes. Representative autoradiograms are shown.

I). In the presence of 10% FCS for 72 h, the expression of c-fos mRNA remarkably increased by threefold as compared to IGF-I or untreated cells. However, the expression of c-myc, β -actin, G6PD, H-ras, and c-raf increased in the presence of 10% FCS in a similar fashion to that in the IGF-I, related to untreated cells (Fig. 7, Table I). Concurrently, the expression of specific differentiation genes, such as ME and UCP present in fetal brown adipocytes before culture (45) (Fig. 1), strongly decreased after 72 h of primary culture in the quiescent-untreated cells. In the presence of IGF-I or 10% FCS, however, the expression of both genes was considerably higher than in the untreated cells after 72 h of culture, although these results were not densitometrically quantified at this stage due to the necessary overexposure of the UCP mRNA expression (Fig. 7).

IGF-I Increased the Expression of the Uncoupling Protein mRNA at 48 and 72 h

A time-course at 24, 48, and 72 h was performed to study the effect of IGF-I, or 10% FCS, on the expression of the UCP mRNA in fetal brown adipocyte primary cultures as compared to untreated cells. Northern blot analysis of total RNA and its densitometric analysis using 18S rRNA for normalization are shown in Fig. 8. As described above, the expression of UCP mRNA drastically decreased between 24 and 48 h of culture in fetal brown adipocytes, remaining almost undetectable at 72 h. The presence of 10% FCS at 48 and 72 h maintained the UCP mRNA expression at almost the same level found at 24 h in the untreated cells. IGF-I, however, increased fivefold the UCP mRNA expression at 48 h and tenfold at 72 h relative to their corresponding control values. The latter increased, even though to a higher level than that found in the IGF-I treated or untreated cells at 24 h of culture (Fig. 8).

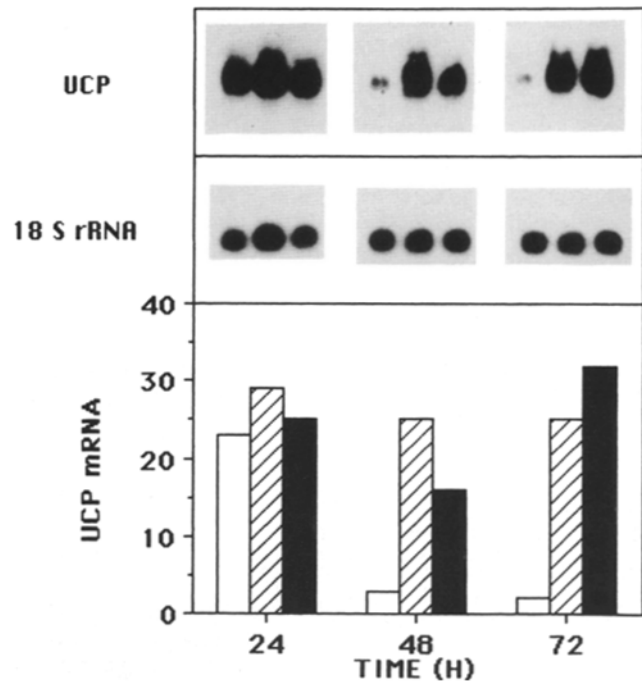


Figure 8. IGF-I increased the expression of the uncoupling protein mRNA at 48 and 72 h. Quiescent cells (20 h serum-starved) were cultured for 24, 48, and 72 h in the presence of IGF-I (1.4 nM) (■), or 10% FCS (▨), or untreated cells (□). Total RNA (20 μ g) was submitted to Northern blot analysis and hybridized with UCP cDNA (upper panel) and 18S rRNA cDNA (central panel). Densitometric analysis of the autoradiograms, after normalization of densitometric units with the amount of 18S rRNA detected, is shown in the lower panel.

Discussion

Our studies show that fetal rat brown adipocytes at time zero of culture constitute a population of cells of broad spectrum, as indicated by cell size, lipid content and endogenous fluorescence, presenting intrinsic mitogenic competence as revealed by the number of cells in S+G₂+M phases of the cell cycle, and constitutively expressing growth-related genes and differentiation-related genes. In addition, fetal brown adipocytes present a high IGF-IR mRNA expression, high IGF-I-binding affinity, and a high number of IGF-I-binding sites per cell. After cell quiescence, our studies also show that IGF-I can behave as mitogen per se and mimic the effect of the fetal serum increasing the DNA synthesis, the percentage of cells into the cell cycle, and the cell number. However, considering that IGF-I-stimulated proliferation lagged behind serum-stimulated growth by 24 h, the possibility cannot be ruled out that IGF-I may induce the expression of endogenously produced growth factors producing a conditioned medium. In addition, single cell analysis of [³H]thymidine-labeled nuclei revealed that some fetal brown adipocytes, showing their typical multilocular lipid droplets distribution, result positive to [³H]thymidine-labeled nuclei in response to IGF-I, suggesting that differentiated cells become proliferative in culture in response to IGF-I.

Moreover, IGF-I, in the absence of other exogenous growth factors present, induces in fetal brown adipocytes the expression of early response genes such as c-fos, c-myc, and H-ras.

These genes are early induced by serum in mouse fibroblasts (15, 31). In a defined medium, PDGF ascertains the expression of c-fos and c-myc but fails to increase the expression of H-ras, an absolute requirement for these cells to progress throughout the G1 to the S phase of the cell cycle, leading to the DNA synthesis (19, 31, 37). However, the presence of IGF-I increases the early expression of H-ras in G0-G1 transition, and H-ras-encoded protein may then mediate the progression of these cells from late G1 to S phase of the cell cycle (1, 27). In addition, in Balb/ 3T3 A31 cells the presence of EGF is also required for the commitment of these cells to DNA synthesis. While PDGF is required for the early expression of c-myc and c-fos, and IGF-I for the early expression of H-ras, only EGF is able to increase all three genes, c-myc and c-fos as an early cell-cycle event, and H-ras as a late cell cycle event (27). Although IGF-I may also increase early response genes in non-fibroblastic mammalian cells (5, 18, 29, 32), no evidence is available regarding the implication of IGF-I, as the only signal exogenously added, in the stimulation of c-fos, c-myc, and H-ras as an early event in these cells. Our results suggest the importance of the expression of c-myc, c-fos, and H-ras as an early event for the IGF-I-stimulated DNA synthesis in non-fibroblastic mammalian cells (brown adipocytes), in the absence of other mitogenic signals exogenously added to the culture medium.

In addition, IGF-I in fetal brown adipocytes produces a late response H-ras mRNA expression in parallel to the expression of c-myc, β -actin, and G6PD, suggesting the importance of the expression of H-ras for the progression of these primary cells into the S phase from late G1 of the cell-cycle, and for the DNA synthesis (26, 31). Although yet unknown, the early biochemical events associated with insulin receptor or IGF-IR upon the ligand binding, the overexpression of insulin receptors in NIH 3T3 cells results in the activation of p21-ras to its GTP active form and the stimulation of the DNA synthesis in a ligand-dependent manner (8). Under our experimental conditions, we cannot rule out that, in our primary brown cells, IGF-I might induce active ras-GTP complex, triggering as an early event the expression of c-fos, c-myc, and H-ras in G0-G1 transition of the cell cycle, and as a late event induces the expression of H-ras and newly encoded-ras protein, allowing cells to progress into the S phase and DNA synthesis, as previously suggested (27).

The present study also shows that IGF-I increases the expression of c-raf in fetal brown adipocytes. The expression of this gene has been proposed as an early event occurring downstream ras activation, in the growth factors signaling transduction pathways involved in mouse fibroblasts proliferation or transformation (9, 40, 49). Our results suggest the importance of the c-raf mRNA late expression in the IGF-I-induced signaling pathways involved in brown adipocytes proliferation.

IGF-I increases proliferation and the expression of the malic enzyme mRNA at 72 h and UCP mRNA in a time-dependent manner in cultured fetal brown adipocytes. We have recently shown that insulin, or IGF-I, or the expression of transfected ras in the absence of insulin/IGF-I, produce 3T3L1 mouse fibroblast differentiation into adipocytes (4). The effect of insulin/IGF-I on 3T3L1 cells increases active ras-GTP complex (35). Accordingly, IGF-I signaling transduction pathways, mediating proliferation in fetal rat brown adipocytes, might be partly involved in the expression of

differentiation-regulated genes, such as ME and UCP mRNA, the activation and/or the expression of ras being a common crucial intermediate.

In conclusion, IGF-I behaves as mitogen per se, as the only growth factor exogenously added to cultured fetal rat brown adipocytes, stimulating DNA synthesis, and increasing the percentage of cells into S+G₂+M phases of the cell-cycle and the cell number, to an extent similar to 10% FCS. In addition, single differentiated brown adipocytes, showing their typical multilocular fat droplets distribution, become positive for [³H]thymidine-labeled nuclei in response to IGF-I. Moreover, IGF-I increases c-fos, c-myc, and H-ras as an early regulatory event, and c-myc, H-ras, c-raf, and G6PD as a late regulatory event, in the absence of other exogenous mitogenic signals. In addition, IGF-I increases the mRNA expression of the lipogenic gene ME and the tissue-specific gene UCP. Our results suggest the involvement of IGF-I in the differentiation as well as in the proliferation processes in primary fetal brown adipocytes.

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References

1. Aaronson, S. A. 1991. Growth factors and cancer. *Science (Wash. DC)*. 254:1146-1153.
2. Alitalo, L., M. Schwab, C. C. Lin, H. E. Varmus, and J. M. Bishop. 1983. Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. *Proc. Natl. Acad. Sci. USA*. 80:1707-1711.
3. Amasino, R. M. 1986. Acceleration of nucleic acid hybridization rate by polyethylene glycol. *Anal. Biochem.* 152:304-307.
4. Benito, M., A. Porras, A. R. Nebreda, and E. Santos. 1991. Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of ras oncogenes. *Science (Wash. DC)*. 253:565-568.
5. Bhat, N. R., K. F. Hausser, and M. S. Kindy. 1992. Cell proliferation and protooncogene induction in oligodendroglial progenitors. *J. Neurosci. Res.* 32:340-349.
6. Bonner, T., S. J. O'Brien, W. G. Nash, and U. R. Rapp. 1984. The human homologs of the raf(mil) oncogene are located in human chromosomes 3 and 4. *Science (Wash. DC)*. 223:71-74.
7. Bouillaud, F., D. Ricquier, J. Thibault, and J. Weissenbach. 1985. Molecular approach to thermogenesis in brown adipose tissue: cDNA cloning of the mitochondrial uncoupling protein. *Proc. Natl. Acad. Sci. USA*. 82:445-448.
8. Burgering, B. M. Th., R. H. Medema, J. A. Maasen, M. L. van de Wetering, A. J. van der Eb, F. McCormick, and J. L. Bos. 1991. Insulin stimulation of gene expression mediated by p21ras activation. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1103-1109.
9. Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell*. 64:281-302.
10. Chomczynski, K., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
11. Cleveland, D., M. A. Lopata, R. J. MacDonald, N. J. Cowand, W. J. Rutter, and M. W. Kirschner. 1980. Number and evolutionary conservation of α - and β -tubulin and cytoplasmic β - and γ -actin genes using specific cloned cDNA probes. *Cell*. 20:95-105.
12. Czech, M. P. 1989. Signal transduction by the insulin-like growth factors. *Cell*. 59:235-238.
13. Deshpande, A. K., and H. F. Kung. 1987. Insulin induction of *Xenopus laevis* oocyte maturation is inhibited by monoclonal antibody against p21

- ras proteins. *Mol. Cell. Biol.* 7:1285-1288.
14. Froesch, E. R., C. Schmid, J. Schwander, and J. Zapf. 1985. Action of insulin-like growth factors. *Annu. Rev. Physiol.* 47:443-467.
 15. Greengard, M. E., and E. B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature (Lond.)* 311:433-438.
 16. Greespan, P., E. P. Mayer, and S. D. Fawles. 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. *J. Cell. Biol.* 100:965-973.
 17. Hartmann, K. K. P., V. Papa, E. J. Brown, U. Doerries, S. M. Rosenthal, and I. D. Goldfine. 1990. A rapid and simple one step method for isolation of poly(A)+ RNA from cells in monolayer. *Endocrinology*. 127:2038-2040.
 18. Hauguel-de Mouzon, S., and C. R. Kahn. 1991. Insulin-like growth-factor-mediated phosphorylation and protooncogene induction in Madin-Darby canine kidney cells. *Mol. Endocrinol.* 5:51-60.
 19. Heldin, C. H., and B. Westermark. 1990. Signal transduction by the receptor for platelet derived growth factor. *J. Cell Sci.* 96:193-196.
 20. Hernandez, E. R., A. Hurwitz, L. Botero, E. Ricciarelli, H. Werner, C. T. Jr. Roberts, D. LeRoith, and E. Y. Adashi. 1991. The insulin-like growth factor receptor gene family in the rat ovary: divergent regulation of distinct receptor species. *Mol. Endocrinol.* 5:1799-1805.
 21. Ho, Y-S., A. J. Howard, and J. D. Crapo. 1988. Cloning and sequence of a cDNA encoding rat glucose 6-phosphate dehydrogenase. *Nucleic Acids Res.* 16:7746.
 22. Kaleko, M., W. J. Rutter, and A. D. Miller. 1990. Overexpression of the human insulin-like growth factor I receptor promotes ligand-dependent neoplastic transformation. *Mol. Cell. Biol.* 10:464-473.
 23. Korn, L. J., C. W. Siebel, F. McCormick, and R. A. Roth. 1987. Ras p21 as a potential mediator of insulin action in xenopus oocytes. *Science (Wash. DC)*. 236:840-843.
 24. Lical, J. C., E. Santos, V. Notario, M. Barbacid, S. Yamazaki, H.-F. Kung, C. Seamans, S. Mc Andrew, and R. Crowl. 1984. Expression of normal and transforming H-ras genes in Escherichia coli and purification of their encoded p21 proteins. *Proc. Natl. Acad. Sci. USA.* 81:5305-5309.
 25. Lorenzo, M., C. Roncero, I. Fabregat, and M. Benito. 1988. Hormonal regulation of rat foetal lipogenesis in brown-adipocyte primary cultures. *Biochem. J.* 251:617-620.
 26. Lu, K., and J. Campisi. 1992. Ras proteins are essential and selective for the action of insulin-like growth factor I late in the G1 phase of the cell cycle in BALB/c murine fibroblast. *Proc. Natl. Acad. Sci. USA.* 89:3889-3893.
 27. Lu, K., R. A. Levine, and J. Campisi. 1989. c-ras-Ha gene expression is regulated by insulin or insulinlike growth factor and by epidermal growth factor in murine fibroblast. *Mol. Cell. Biol.* 9:3411-3417.
 28. Magnuson, M. A., and V. M. Nikodem. 1983. Molecular cloning of a cDNA sequence for rat malic enzyme. *J. Biol. Chem.* 258:12712-12717.
 29. Merriman, H. L., D. LaTour, T. A. Linkhart, S. Mohan, D. J. Baylink, and D. D. Strong. 1990. Insulin-like growth factor-I and insulin-like growth factor II induce c-fos in mouse osteoblastic cells. *Calcif. Tissue Int.* 46:258-262.
 30. Mosley, S. T., J. L. Goldstein, M. S. Brown, J. R. Flack, and R. G. W. Anderson. 1981. Targeted killing of cultured cells by receptor dependent photosensitization. *Proc. Natl. Acad. Sci. USA.* 78:5717-5721.
 31. Mulcahy, L. S., M. R. Smith, and D. W. Stacey. 1985. Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. *Nature (Lond.)*. 313:242-243.
 32. Pavelic, K., N. P. Slaus, and R. Spaventi. 1991. Growth factors and proto-oncogenes in early mouse embryogenesis and tumorigenesis. *Int. J. Dev. Biol.* 35:209-214.
 33. Phillips, P. D., R. J. Pignolo, and V. G. Cristofalo. 1987. Insulin-like growth factor: specific binding to high and low affinity sites and mitogenic action throughout the life span of WI-38 cells. *J. Cell. Physiol.* 133:135-143.
 34. Pietrzowski, Z., R. Lammers, G. Carpenter, A. M. Soderquist, M. Limardo, P. D. Phillips, A. Ullrich, and R. Baserga. 1992. Constitutive expression of insulin-like growth factor I and insulin-like growth factor I receptor abrogates all requirements for exogenous growth factors. *Cell Growth & Differ.* 3:199-205.
 35. Porras, A., A. R. Nebreda, M. Benito, and E. Santos. 1992. Activation of Ras by insulin in 3T3 L1 cells does not involve GTPase-activating protein phosphorylation. *J. Biol. Chem.* 267:21124-21131.
 36. Ricquier, D., F. Bouillaud, P. Toumelin, G. Morg, R. Bazin, J. Arch, and L. Penicaud. 1986. Expression of the uncoupling protein mRNA in thermogenic or weakly thermogenic brown adipose tissue. *J. Biol. Chem.* 261:13905-13910.
 37. Rozengurt, E. 1986. Early signals in the mitogenic response. *Science (Wash. DC)*. 234:161-166.
 38. Rozengurt, E. 1991. Measurement of the proliferative effects of cytokines. In *Cytokines: A Practical Approach*. F. R. Balkwill, editor. IRL Press at Oxford University Press, Oxford. 253-259.
 39. Rubin, C. S., A. Hirsch, C. Fung, and O. M. Rosen. 1978. Development of hormone receptors and hormonal responsiveness in vitro. *J. Biol. Chem.* 253:7570-7578.
 40. Santos, E., and A. R. Nebreda. 1989. Structural and functional properties of ras proteins. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:2151-2163.
 41. Schwartz, D. E., R. Tizard, and W. Gilbert. 1983. Nucleotide sequence of rous sarcoma virus. *Cell.* 32:853-869.
 42. Surmacz, E., P. Nugent, Z. Pietrzowski, and R. Baserga. 1992. The role of the IGF1 receptor in the regulation of cdc2 mRNA levels in fibroblasts. *Exp. Cell Res.* 199:275-278.
 43. Thorell, B. 1983. Flow-cytometric monitoring of intracellular flavins simultaneously with NAD(P)H levels. *Cytometry.* 4:61-65.
 44. Valverde, A. M., M. Benito, and M. Lorenzo. 1991. Proliferation of fetal brown adipocyte primary cultures: relationship with the genetic expression of glucose 6 phosphate dehydrogenase. *Exp. Cell Res.* 194:232-237.
 45. Valverde, A. M., M. Benito, and M. Lorenzo. 1992. Hormonal regulation of malic enzyme and glucose-6-phosphate-dehydrogenase expression in fetal brown-adipocyte primary cultures under non-proliferative conditions. *Eur. J. Biochem.* 203:313-319.
 46. Van Straaten, F., R. Müller, T. Curran, C. Van Beveren, and I. M. Verma. 1983. Complete nucleotide sequence of a human c-oncogene: deduced amino acid sequence of the human c-fos protein. *Proc. Natl. Acad. Sci. USA.* 80:3183-3187.
 47. Werner, H., B. Stannard, M. A. Bach, D. LeRoith, and C. T. Jr. Roberts. 1990. Cloning and characterization of the proximal promoter region of the rat insulin-like growth factor I (IGF-I) receptor gene. *Biochem. Biophys. Res. Commun.* 169:1021-1027.
 48. Werner, H., M. Woloschak, M. Adamo, Z. Shen-Orr, C. Jr. Roberts, and D. LeRoith. 1989. Developmental regulation of the rat insulin-like growth factor I receptor gene. *Proc. Natl. Acad. Sci. USA.* 86:7451-7455.
 49. Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis. 1992. ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1 and RSK. *Cell.* 68:1041-1050.