FEBS Letters 354 (1994) 232–236

FEBS 14746

Regulatory effects of galactose on galactose-1-phosphate uridyltransferase activity on human hepatoblastoma HepG2 cells

A. Davit-Spraul[b,*], M.L. Pourci[a,b], K. Heng Ng[c], T. Soni[a], A. Lemonnier[a,b]

[a]*Laboratoire de Biochimie Appliquée, Faculté de Pharmacie-Paris-XI, 92296 Chatenay-Malabry, France*
[b]*Laboratoire Central de Biochimie, Hôpital Bicêtre, 78, rue du Général Leclerc, 94275 Le Kremlin Bicêtre, France*
[c]*INSERM U347, 80 rue du Général Leclerc, 94276 Le Kremlin-Bicêtre Cedex, France*

Received 30 August 1994; revised version received 3 October 1994

Abstract Galactose-1-phosphate uridyltransferase (GALT) deficiency results in galactosemia in man. We have studied the regulation of the GALT gene expression on the HepG2 cell line by growing the cells in glucose or galactose medium. No difference of K_m values was observed in glucose or galactose media but the V_{max} value with galactose was 50% higher than that with glucose. Also in galactose medium, an increased GALT specific activity was detected suggesting the production of more enzyme proteins. Yet, slot dot quantification of GALT mRNA revealed a decreased amount of these transcripts in cells cultured with galactose or inosine while Northern blot analysis revealed the normal 1.4 kb transcript in all culture media used. Finally, IEF gel analysis displayed different isozymic patterns for the GALT enzyme in cells grown in glucose, galactose or inosine media. With glucose-free media, the major band of GALT corresponds to that found in human liver. Altogether, these results suggest that the control of GALT gene expression in HepG2 cells is located at the post-transcriptional level and correlated to the growth rate of the cell.

Key words: Galactose-1-phosphate uridyltransferase; HepG2 cell

1. Introduction

Galactose-1-phosphate uridyltransferase (GALT: EC 2.7.7.12) catalyzes an essential step in galactose catabolism in which galactose-1-phosphate is converted to glucose-1-phosphate. The absence of this enzyme catalytic activity in man results in galactosemia, an autosomal recessive disorder [1] in which the liver is the target organ for galactose toxicity. An important progress in the comprehension of this dysfunction has been made recently due to the cloning of the GALT cDNA by Reichardt and Berg [2] which has permitted the identification of a variety of mutations in the GALT gene correlated to the disease [3–5]. Finally, recent data [6] demonstrated an interrelationship between the post-natal rat liver GALT mRNA and a rise and fall of GALT specific activities.

The regulation of GALT activity has been extensively studied by perfusing suckling rat liver with galactose or glucose. Indeed, the authors [7] found that the perfusion (recirculating mode) for 30 min with galactose or glucose resulted in a 50% increase of transferase specific activity followed by a precipitous decline [8]. No such changes occurred in perfused adult rat livers or by single pass perfusion in suckling rats [9]. The authors suggested that the effects observed were due to the metabolic product common to glucose and galactose. In rats fed on a high galactose diet [10,11], GALT specific activity was increased. This effect was also observed for pregnant and suckling rats whereas an inverse change was induced by lactation and in the fetus [12]. All these results demonstrate that GALT specific acivity can be modulated by galactose or its metabolites and possibly by the hormonal status.

In a previous article, we reported the effects of galactose on the regulation of galactose metabolizing enzymes in HepG2 cells, an established human hepatoma line in which most liver-specific functions are expressed. Our results showed that the substitution of glucose by galactose in the culture medium decreased the specific activity of galactokinase (GALK) and increased that of GALT [13]. Based on these observations, we undertook a more detailed investigation of GALT gene expression by studying the kinetic characteristics of this enzyme, its isoenzyme profile and the modulation of GALT mRNA amounts in HepG2 cells grown in galactose medium.

2. Materials and methods

All unlabelled chemicals were reagent grade from Boehringer-Mannheim (Germany); Merck (Darmstadt, Germany) or Sigma (St. Louis, Missouri, USA). Radioisotopes were purchased from Amersham (UK). Culture medium (RPMI) and fetal calf serum were supplied by Gibco (France).

2.1. Cell culture conditions

The standard procedure for HepG2 cells culture consisted of growth in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamin, 20 UI/ml penicillin and 20 mg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator. Every week cells were trypsinized, diluted six-fold and seeded (3×10^6 cells) in 75 cm^2 plastic tissue culture flasks (Falcon). The medium was changed every 2 days.

To study the effects of galactose, HepG2 cells were cultured in hexose-free RPMI 1640 medium supplemented with 11 mM galactose, 10% dialysed FCS (48 h dialysis against 0.15 M NaCl), antibiotics and glutamin as indicated above. The cells were used on day 7 during the exponential growth phase. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) before biochemical characterization.

2.2. Enzyme assays

Cells were frozen at −80°C, thawed and harvested by scraping with a rubber policeman. Lysed cells were then suspended in water containing 10 mM dithiothreitol (DTT). After centrifugation at 4°C, the 800*g* supernatant was removed for enzyme and protein assays.

GALT was assayed by the technique previously described and modified for HepG2 cells [14]. The reaction mixture contained 1 mM [U-^{14}C]galactose-1-phosphate (0.1 μCi), 0.2 mM UDP glucose, 100 mM glycine-NaOH (pH 8.2), 10 mM DTT and 0.5 g/l cell lysate proteins. The labelled products were separated by chromatography on DEAE-cellulose paper (Whatman DE 81) and eluted with lithium chloride according to the method of Ng et al. (15). All assays were performed in duplicate. Specific activities were expressed as nmol of product formed/min/mg of soluble protein.

*Corresponding author. Fax: (33) 45213574.

SSDI 0014-5793(94)01133-8

Protein was assayed with a BioRad kit (New York, USA) with bovine serum albumin as the standard.

2.3. Isoelectrofocusing (IEF) patterns of isoenzymes

Cells were lysed by the freeze and thaw method, collected by scraping and suspended in a solution containing 10 mM DTT and 4.8 mM UDP-glucose. After centrifugation at 4°C, the 800*g* supernatant was concentrated on Amicon to 60 mg soluble proteins per sample. IEF was then carried out using the Phast System (Pharmacia) according to the method of Brivet et al. [16].

2.4. RNA isolation and analysis

Total cellular RNA was isolated by extraction with RNAsol (Bioprobe Systems, France) according to the conditions of the supplier. The GALT cDNA used as a probe was kindly provided by Dr. J. Reichardt.

For Northern blot analysis, 30 μg of total RNA from HepG2 cells cultivated in different media were fractionnated on a 1% agarose-formaldehyde gel according to Maniatis et al. [17]. After transfer of the RNA on a nylon membrane (Hybond N, Amersham), the filter was baked 2 h at 80°C, prehybrized in a cocktail solution (5 × SSC, 5 × Denhardt's solution, 0.5% SDS and 100 μg/ml salmon sperm DNA) at 42°C for 2 h and then hybridized overnight at 42°C in the same cocktail solution containing the nick-translated ^{32}P-GALT cDNA (Boehringer-Mannheim kit). The filter was washed to a final stringency of 1 × SSC, 0.1% SDS at 65°C for 30 min and autoradiographed with Kodak XAR-5 film between intensifying screens. The same nylon membrane was dehybridized and then hybridized with a ^{32}P-β-actin probe [18] in order to quantify the relative amounts of GALT mRNA in the different samples.

Slot dot analysis was done with 2.5 μg, 5 μg and 10 μg of total RNA per sample with Hybond N membrane and treated as described above with the radiolabelled probes. Autoradiography was carried out in the absence of intensifying screens. Total RNAs were quantified by spectrophotometry and densitometry.

3. Results and discussion

3.1. Kinetic characteristics

GALT specific activities presented in Table 1 were measured on HepG2 cells grown in glucose or galactose media. The results show a 50% increase of the GALT enzyme activity in cells grown in galactose (12 nmol/min/mg protein) as compared to those grown in glucose medium (8 nmol/min/mg protein).

The relationships between substrate concentrations and GALT activities are illustrated in Fig. 1. No difference was observed in the K_m of enzyme with galactose-1-phosphate as the substrate in the presence of 0.2 mM UDP-glucose (Fig. 1a) for cells grown in galactose (K_m: 0.10 mM) or those grown in glucose (K_m: 0.09 mM). The same observation was noted with UDP-glucose as the substrate in the presence of 1 mM galactose-1-phosphate; the K_m for cells cultured in glucose medium was 0.11 mM compared to 0.12 mM for cells cultured in galactose medium (Fig. 1b). However, high concentrations of galactose-1-phosphate (> 1.5 mM) or UDP-glucose (> 0.5 mM) inhibited GALT specific activities in cells grown in either medium. These results indicate that the GALT proteins produced by cells grown in both types of culture media have the same affinity for its substrates.

The V_{max} were affected by the culture conditions as the values for cells grown in galactose or glucose are different. Indeed, HepG2 cells grown in galactose presented V_{max} of 14 nmol/min/mg protein with the galactose-1-phosphate and 21 nmol/min/mg protein with UDP-glucose whereas those grown in glucose presented, respectively, 10 nmol/min/mg protein and 12 nmol/min/mg protein. This result indicates that even though the affin-

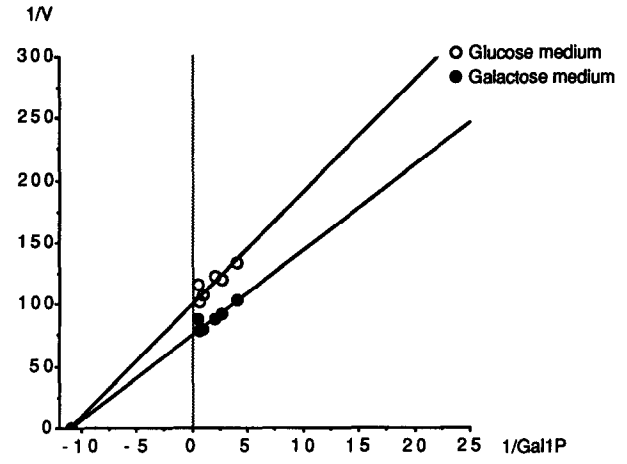

Fig.1 a

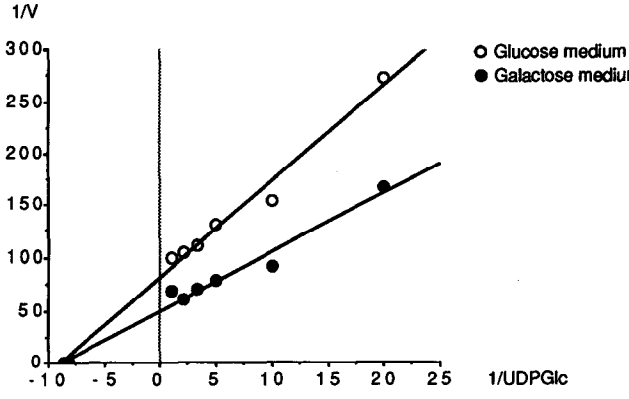

Fig. 1 b

Fig. 1. Interrelationship of GALT activities and its substrates. (a) Substrate: galactose-1-phosphate. The K_m values were measured in the presence of 0.2 mM UDP-glucose. (b) Substrate: UDP-glucose. The K_m values were measured in the presence of 1 mM galactose-1-phosphate. Regression analysis was used to calculate the lines shown from which data exhibiting substrate inhibition were excluded. Specific activities (v) are expressed as nmol of product formed/min/mg soluble enzyme. The concentration of substrates are mmol/l.

Table 1
Comparison of relative amounts of GALT mRNA and specific activities of GALT

Medium	Actin	GALT	GALT/ Actine	Sp.act GALT
Glucose	1	1	1	8
Galactose	0,85	0,43	0,51	12
Galactose + inosine	0,93	0,35	0,38	nd
Inosine	0,47	0,15	0,31	nd

nd: non determined.
Slot blot experiments were performed on total cellular RNAs using successively ^{32}P-labeled GALT cDNA and actin cDNA as probes. Films were scanned and the hybridization intensity was integrated. Values of GALT and actin are expressed relative to the GALT and actin values for glucose condition considered as 1.
Specific activities of GALT are expressed in nmol/min/mg.

ities of the enzymes did not differ under different culture conditions, the rate of enzymatic activity increases by at least 40% in cells grown in galactose as compared to those grown in glucose. This increased V_{max} for the two substrates and the enhanced specific activity suggest that more GALT proteins may be produced in HepG2 cells under the influence of galactose. This hypothesis is the same as proposed by Rogers et al. [11] who also reported an increased GALT activity in liver of rats fed on a galactose diet which was associated with an increased V_{max} for UDP-glucose but a similar K_m as compared to control rats.

The enhancement of GALT activity induced by galactose could be explained by the increased production of enzyme proteins which could be correlated with the production of more RNA molecules. So, we undertook the qualitative and quantitative analyses of GALT mRNA by Northern and slot blots.

3.2. RNA analyses by Northern and slot blots

Total RNA were isolated from HepG2 cells grown in glucose or galactose as well as in inosine, a non-hexose source, or galactose supplemented with inosine.

Fig. 2-I represents a Northern blot of HepG2 mRNA prepared from cells grown in different media and probed with ^{32}P-labelled GALT cDNA (Fig. 2-Ia) or β-actin cDNA (Fig. 2-Ib). Only one band of 1.4 kb corresponding to the size of GALT mRNA was observed in cells grown in galactose (lane 1), galactose plus inosine (lane 2), glucose (lane 3) or inosine (lane 4). This result indicates that the same molecular form of the GALT mRNA are present in cells grown in the different culture media.

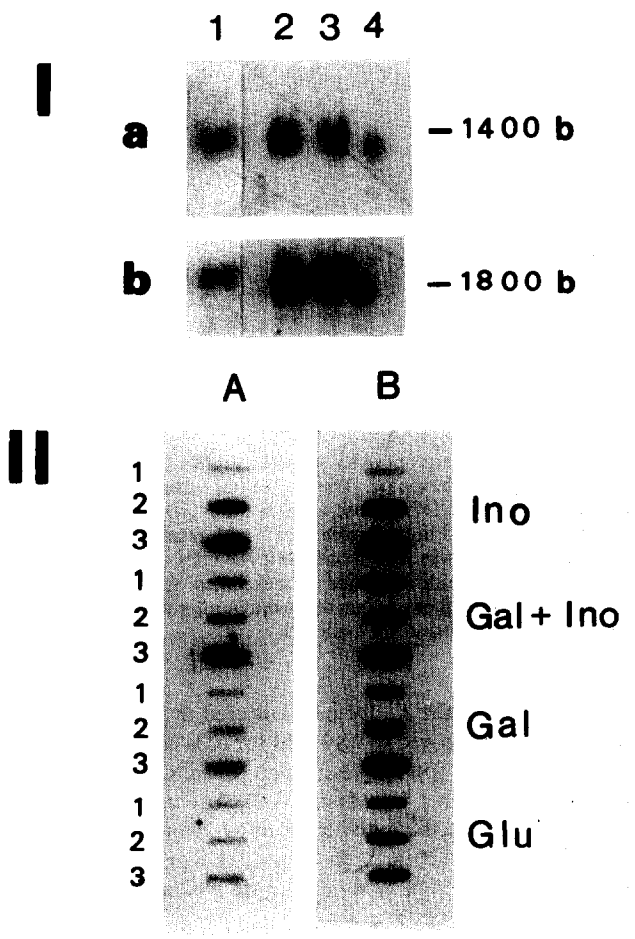

Fig. 2. Northern (I) and slot (II) blot analyses of RNA from HepG2 cells grown in different culture media. (I) Northern blot of total RNA (30 μg/lane) extracted from HepG2 cells grown in galactose (lane 1), galactose + inosine (lane 2), glucose (lane 3) and inosine (lane 4). Probes used were (a) human GALT cDNA and (b) mouse β-actin cDNA. (II) Slot blot analysis with 2.5 μg (slot 1), 5 μg (slot 2) and 10 μg (slot 3) of total RNA. Panel (A) was hybridized with GALT cDNA and Panel (B) with β-actin cDNA.

In order to analyse the quantitative effects of culture medium on GALT mRNA amount, a slot blot was performed on the different RNA samples and probed with radiolabelled GALT (Fig. 2-IIA) or β-actin (Fig. 2-IIB) cDNAs. The values obtained by densitometric quantification of the GALT mRNA signals standardized to those of actin are presented in Table 1. The results reveal that (i) glucose leads to the production of more GALT transcripts, (ii) the amounts of these transcripts are greatly reduced by growth in the three glucose-free media, (iii) inosine affects GALT mRNA amounts as with galactose and (iv) there is no synergistic effect by adding inosine to galactose. These observations remain valid even when the signals of GALT mRNA are standardized to actin gene expression. Indeed, the relative amounts of GALT transcripts in cells cultured with galactose (0.51) or inosine (0.31) are reduced by at least half as compared to cells grown with glucose (1.00). Therefore, this quantitative RNA analysis indicates that the enhanced GALT activity induced by galactose is not supported by an increment in the mRNA.

A report by Heidenreich et al. [6] showed a correlation between GALT specific activity and steady state mRNA in various tissues of the adult rat and during postnatal development. Therefore, galactose or its metabolites were suggested by these authors to be regulators of this developmental modulation for both increasing RNA transcription and enzyme activity in postnatal rat liver due to the large amounts of galactose, via lactose, present in milk. Contrary to the above data, our results showed decreased GALT mRNA in HepG2 cells grown in galactose medium. The discrepancy between the response of postnatal rat liver and HepG2 cells to high amounts of galactose could be due to species specificity or to different experimental models. Several reasons can also account for this observation such as (i) a curtailed trancription rate, (ii) less stable RNA molecules resulting in more rapid degradation, or (iii) a faster translation process in protein production. The increased amount of enzyme proteins suggested by the rise of the V_{max} value could be due to the generation of a more stable protein molecule.

In order to test the hypothesis of post-translational modifications of the GALT enzyme due to galactose effects, we undertook the analysis of isozymic forms of the GALT enzyme by IEF gel electrophoresis.

3.3. GALT isozymic pattern

IEF gel electrophoresis between pH 4 and 6.5 had demonstrated a microheterogeneity of the GALT protein with multiple banding patterns between different tissues [19]. As shown in Fig. 3a, human liver GALT exhibited 5 activity bands with band 3 as the major one. HepG2 cells cultured in glucose (Fig. 3b) produced GALT proteins which exhibited only 4 of these bands situated near the cathode, the major band corresponding to band 4 of human liver DNA. GALT proteins induced by 2.5 mM inosine, a ribose donor, are also composed of 4 activity bands but situated near the anode (Fig. 3d) whereas the pres-

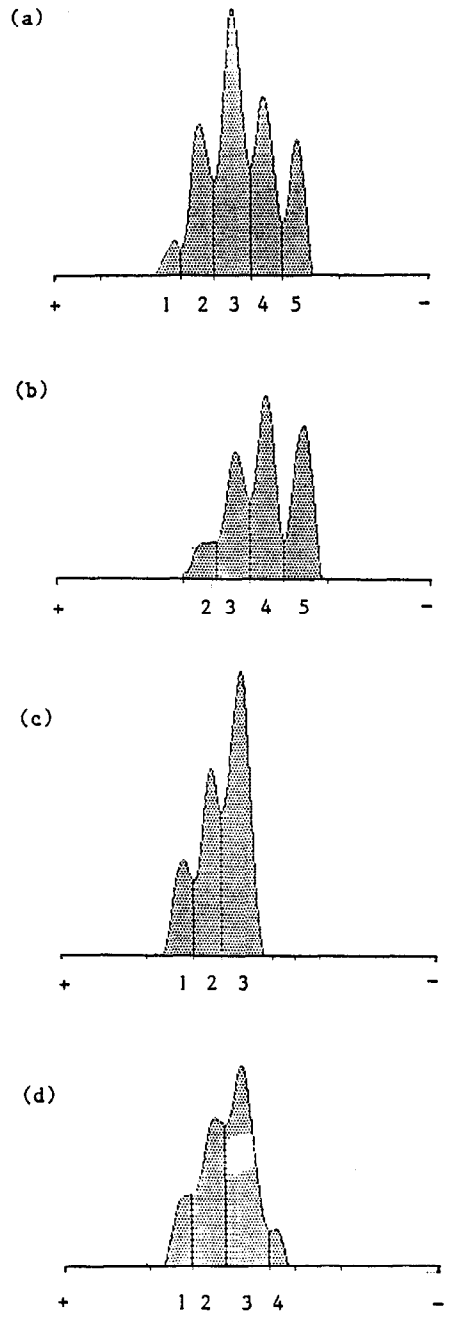

Fig. 3. GALT isozyme patterns of HepG2 cells grown in different media. HepG2 cells were grown in different culture media and extracted proteins were analysed by high resolution IEF polyacrylamide gel (pH 4–6.5). The gel was stained enzymatically for transferase and the bands were scanned to integrate their relative density. (a) Human liver, (b) glucose, (c) galactose and (d) inosine.

ence of galactose gave 3 bands also situated near the anode (Fig. 3c). However, the most important activity band induced by galactose or inosine corresponds to band 3 in human liver. Although the presence of galactose does influence the microheterogeneity of the GALT enzyme, this effect is cancelled once glucose is supplemented to the galactose medium and the IEF profile is the same as that found for glucose alone (data not shown). This result reflects published data [11] in which the band activities for hepatic transferase in control rats and those fed on a galactose enriched diet were similar, thus indicating the absence of the galactose effect. Therefore the results from IEF gel elctrophoresis suggest a correlation between the major band 3 and increased GALT activity found in cells cultured with galactose.

The 'anodisation' of the GALT protein does not seem to be specific for galactose as inosine produces the same effect. Indeed, the changes in IEF isozymic patterns could be the consequence of a slower growth rate as the doubling time for HepG2 cells cultured in galactose or inosine is twice that of cells grown in glucose medium [13]. This hypothesis is also proposed by Kelley et al. [19] on their work with cultured fibroblasts who suggested that the anodisation of the GALT pattern between the exponential and stationary phases could be related to a slower growth rate. Other data by Schapira et al. [20] on the comparison of isoelectrofocusing patterns of transferase in mature red blood cells (erythrocytes) with those of the precursors, reticulocytes and erythroblasts, demonstrated a progressive anodisation of bands from erythroblasts to erythrocytes. The pH_i of red blood cell transferase is much more anodic than those of other tissues and corresponds to the most anodic band found in liver. The authors proposed post-translational mechanisms such as glycosylation or deamidation to be involved in cells with a longer turn-over and suggested that the microheterogeneity could be due to post-synthetic and progressive modifications of the charges in the subunits as a consequence of their aging. However, GALT is not described to be a glycosylated protein. Therefore, the differences in IEF patterns may be supported by the latter supposition of modifications in ionic charges resulting in more stable protein forms due to a longer turn-over of cells influenced by culture conditions. GALT proteins in HepG2 cells which grow slowly such as those in galactose or inosine are able to undergo complete maturation thus producing the isoform involved with the most enzyme activity whereas rapidly dividing cells grown in glucose may produce more enzyme proteins but lack the time necessary for complete maturation of these molecules.

In conclusion, galactose in the culture medium of HepG2 cells does not have a specific effect on the expression of the GALT enzyme. Our results indicate that the rise in galactose-induced GALT activity is not correlated to the presence of more enzyme proteins as a consequence of enhanced transcription of the mRNA but is correlated to the existence of specific isozymic forms of the GALT protein as the result of slow cell growth. In spite of the elimination of galactose from the diet, patients with transferase deficiency present mental retardation, ovarian failure and late neurological abnormalities as long term effects. Thus an understanding of the regulatory mechanisms enhancing transferase activity might contribute to the augmentation of the residual transferase activity as a possible therapeutic strategy.

Acknowledgements: We are indebted to Dr. J. Deschatrette for critical reading of this manuscript.

References

[1] Segal, S. (1991) in: Metabolic basis of inherited diseases (Stanbury, J.B. Ed.) Chapter 13, pp. 453–480, McGraw-Hill, New York.
[2] Reichardt, J.K.V. and Berg, P. (1988) Mol. Biol. Med. 5, 107–122.
[3] Reichardt, J.K.V. and Woo, S.L.VC. (1991) Prod. Natl. Acad. Sci. USA 88, 2633–2637.

[4] Reichardt, J.K.V., Levy, H.L. and Woo, S.L.V.C. (1992) Genomics, 12, 596–600.
[5] Reichardt, J.K.V, Belmont, J.W., Levy, H.L. and Woo, S.L.V.C. (1992) Biochemistry 31, 5430–5433.
[6] Heidenreich, R.A., Mallee, J., Rogers, S. and Segal, S. (1993) Ped. Res. 34, 416–419.
[7] Berman, W., Rogers, S.R., Bautista, J. and Segal, S. (1979) Am. J. Physiol. 236, 633–637.
[8] Rogers, S.R. and Segal, S. (1981) Am. J. Physiol. 240, 333–339.
[9] Rogers, S.R. and Segal, S. (1984) Biochem. Med. 31, 112–121.
[10] Mangeot, M., Djabal, A., Myara, I., Cherruau, B., Pourci, M.L. and Lemonnier, A. (1987) Ann. Nutr. Metab. 31, 333–341.
[11] Rogers, S.R., Bovee, B.W., Saunders, S.L. and Segal, S. (1989) Metabolism 38, 810–815.
[12] Rogers, S.R., Bovee, B.W., Saunders, S.L. and Segal, S. (1989) Ped. Res. 25, 161–166.
[13] Davit-Spraul, A., Pourci, M.L., Soni, T. and Lemonnier, A. (1994) Metabolism 43, 945–952.
[14] Segal, S. and Rogers, S.R. (1971) Biochem. Biophys. Acta 250, 351–360.
[15] Ng, W.G., Bergren, W.R. and Donnell, G.N. (1967) Clin. Chim. Acta. 15, 489–492.
[16] Brivet, M., Baptista, J., and Lemonnier, A. (1989) 27th Annual Meeting of SIEM, Munnich.
[17] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, New York.
[18] Minty, A.J., Alonso, S., Guenet, J.L.and Buckingham, M. (1983) J. Mol. Biol. 167, 77–101.
[19] Kelley, R.I., Harris, H.W. and Mellman. (1983) Hum. Genet. 63, 274–279.
[20] Schapira, R., Gregori, C. and Banroques, J. (1978) Biochem. Biophys. Res. Commun. 80, 291–297.