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Control of expression of the gene for the arginine transporter Cat-1 in rat liver cells by glucocorticoids and insulin

J. Liu and **M. Hatzoglou**

Department of Nutrition, Case Western Reserve University School of Medicine, Cleveland, Ohio, U.S.A.

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Summary. Hepatic arginine and lysine uptake is partly regulated by changes in the transport activity of a group of cell surface proteins exhibiting properties of the transport system y^+. The *Cat-1* gene encodes a sodium-independent high-affinity cationic amino acid transporter of the y^+ system which is nearly undetectable in the quiescent liver. In this paper we investigate the regulation of expression of Cat-1 in the quiescent rat liver by glucocorticoids and insulin, two hormones which play a critical role in amino acid dependent pathways of hepatic metabolism. Injection of insulin and glucocorticoids resulted in a rapid (15–30 min, 4–5 fold) increase in transcription which returned to basal levels within 4 hours. In contrast to the rapid single peak of transcriptional induction of the *Cat-1* gene, the accumulation of the Cat-1 mRNAs occurred transiently with two peaks, the first at 30 minutes and the second at 2–4 hours following hormone treatment. These data indicate that expression of the *Cat-1* gene in the quiescent liver can be transiently induced by both transcriptional and post-transcriptional mechanisms. In FTO2B rat hepatoma cells, expression of the gene is constitutive and accumulation of Cat-1 mRNAs in response to dexamethasone and insulin was dependent on transcription and protein synthesis. Furthermore, the accumulation of the basal level of the Cat-1 mRNAs was reduced by 70%, upon treatment of cells with inhibitors of protein synthesis for 6 h, when the transcription rate of the gene did not decrease significantly. We conclude the following: (i) under normal physiologic conditions, expression of the *Cat-1* gene in the quiescent liver is negligible, propably to prevent unnecessary transport and metabolism of arginine by the hepatic arginase in the hepatocytes. (ii) in the cases when hepatic cationic amino acid transport is needed, such as following feeding, cellular growth and illness, glucocorticoids and insulin induce expression of the *Cat-1* gene in liver cells through induction of transcription and stabilization of the mRNA. (iii) constitutive Cat-1 mRNA accumulation in rat hepatoma cells depends on protein synthesis through a labile regulated factor. Overall, constitutive expression of Cat-1 is associated with hepatic cellular growth and transformation.

Keywords: Amino acids – Ecotropic – Receptor – Amino acid transport – Protein synthesis inhibitors

Abbreviations: ActD, actinomycin D; Cat-1, cationic amino acid transporter 1; cx, cycloheximide; dex, dexamethasone; gapdh, glyceraldehyde-3-phosphate dehydrogenase; *PEPCK*, phosphoenol pyruvate carboxykinase; *TAT*, tyrosine amino transferase

Introduction

The supply of amino acids for intermediary metabolism during fasting or feeding is largely controlled by the activity of specific amino acid transporters on the plasma membrane of mammalian cells (White, 1985; White et al., 1984; Christensen, 1990; Malandro et al., 1996). These transport activities function to promote either entry or release of amino acids from cells. A large number of transport systems have been studied regarding their biochemical properties such as kinetics, substrate specificity and cell type specific regulation of their activities (Malandro et al., 1996). The Na^+-independent carrier y^+ is the principle transport system for cationic amino acids in mammalian cells (White, 1985).

Four related proteins with y^+ activity have been isolated, Cat-1, Cat-2 (also known Cat2B), Cat-2a (also known Cat2A) and Cat-3 (Wang et al., 1991; Kim et al., 1991; MacLeod et al., 1990; Closs et al., 1993; Closs et al., 1993; Hosokawa et al., 1997; Ito et al., 1997). All four proteins function as y^+ system transporters for the cationic amino acids arginine, lysine and ornithine, but they differ in their affinity for substrate with the Cat-1, Cat-2 and Cat-3 having 10 times higher substrate-affinity than Cat-2a (Kakuda et al., 1993; Hosokawa et al., 1997). The biological significance of having multiple transporters for cationic amino acids in mammalian cells is not known, but their tissue-specific expression patterns suggest that different members may facilitate amino acid flux in mammalian cells in response to specific nutritional needs. The low affinity-high capacity transporter Cat-2a has a Km 10 times higher than the physiologic concentration of amino acids in the plasma, and it is the only transporter expressed in the quiescent rat liver (Closs et al., 1993). However, reassessment of kinetic data in the literature for arginine transport by the Cat-2a protein showed a low as well as a high Km component (Van Winkle et al., 1995). In agreement with the lack of functional *Cat-1* gene expression in the liver (Wu et al., 1994), are the earlier studies by Christensen and coworkers who have shown that normal hepatocytes lack high affinity y^+ transport activity (White et al., 1982). The absence of Cat-1 from the normal liver restricts entry of the plasma arginine in the hepatocytes, thus protecting the plasma arginine pool from degradation by the hepatic arginase (Kakuda et al., 1993). Therefore, the regulation of expression of the *Cat-1* gene may become the site of control of arginine catabolism by the quiescent liver. However, other reports in the literature support that normal liver hepatocytes contain at low levels a high affinity transport system for cationic amino acids (Espat et al., 1996). It is therefore possible that different systems operate

during the feeding cycle in order to maintain normal plasma amino acid levels. Identification of the factors and the mechanism of regulation of *Cat-1* gene expression will be an important step in our understanding of cationic amino acid transport in hepatocytes.

Cationic amino acid transport is important in the quiescent liver during critical illness and in the growing liver during regeneration (Wu et al., 1994). Induction of *Cat-1* gene expression or hepatic arginine transport has been shown in hepatoma cells (White et al., 1982), the regenerating liver (Wu et al., 1994), in the tumor bearing rat (Inoue et al., 1995; Espat et al., 1994 and 1995) and in response to several mediators of the septic response (Inoue et al., 1995). Souba and coworkers have very elegantly studied hepatic amino acid uptake during various catabolic states (Fischer et al., 1995; Pacitti et al., 1992). They demonstrated that cytokines and TNFα work with glucocorticoids to stimulate, among others, y^+ amino acid uptake in hepatocytes (Fischer et al., 1996; Watkins et al., 1994; Inoue et al., 1994). In cancer and sepsis patients, this accelerated hepatic uptake supports hepatic gluconeogenesis, acute phase protein synthesis, NO production and other key metabolic pathways in the liver. The latter group has concluded that the rapid increase in hepatic amino acid transport that occurs during endotoxemia requires participation of glucocorticoids (Inoue et al., 1995).

The importance of Cat-1 in growth and differentiation has recently been demonstrated (Perkins et al., 1997). Homozygous knock-out mice for Cat-1 were 25% smaller in size, developed severe anemia at mid-gestation and did not survive after birth. Fetal liver from the knock-out mice generated anemia when transplanted to lethally irradiated mice, suggesting that Cat-1 is required for erythropoiesis.

We have suggested that rapid and efficient transport of cationic amino acids in the quiescent liver occurs through careful modulation of expressi on of the *Cat-1* gene (Wu et al., 1994; Aulak et al., 1996; Hatzoglou et al., 1995). It is therefore important to study the regulators of expression of Cat-1 in order to understand the way that the liver controls amino acid availability for key metabolic processes. Our hypothesis was that the hepatic *Cat-1* gene and the genes involved in amino acid-dependent metabolic processes share common regulatory features. In this paper, we investigate the mechanism of regulation of expression of Cat-1 in the quiescent liver by glucocorticoids and insulin. As mentioned above, glucocorticoids are important for hepatic amino acid uptake during catabolic states (Inoue et al., 1995). Furthermore, glucocorticoids regulate the expression of ornithine decarboxylase which is involved in the synthesis of polyamines during cellular growth (Heby et al., 1990), the acute phase response genes (Bauman et al., 1989; Pacitti et al., 1993), the urea cycle enzymes and the hepatic inducible nitric oxide synthase (Geller et al., 1993). The importance of glucocorticoids on the growth of primary hepatocytes in culture has also been established (Block et al., 1996).

Insulin is a key regulator of energy balance. In the liver, it induces protein synthesis, hepatic amino acid transport and has growth promoting effects (for a review, see O'Brien et al., 1996). It is therefore possible that insulin is a

physiologic mediator of transient induction of Cat-1 gene expression in the liver following a meal, to assist hepatic amino acid uptake.

We propose that expression of Cat-1 in the quiescent liver is adaptive to provide cationic amino acids when amino acid transport becomes important, such as starvation, trauma, sepsis, during cell growth or following a meal. We suggest that part of this adaptive response is the transient transcriptional induction of Cat-1 by glucocorticoids and insulin followed by mRNA stabilization.

Materials and methods

Chemicals and reagents

All DNA modifying enzymes and nucleotides were purchased from Boehringer Mannheim. [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from DuPont-New England Nuclear. Restriction enzymes were used as specified by the manufacturer. Actinomycin D, puromycin, cycloheximide and insulin were purchased from Sigma. Dexamethasone Sodium Phosphate was purchased from Lyphomed TM, USA. Rats were purchased from Zivic Miller.

Plasmids and DNA hybridization probes

The following probes were used in this study: (i) *rat Cat-1/2.5*: a 2.5 kb at the 5′-end of the rat Cat-1 cDNA (Wu et al., 1994) (ii) *rat Cat-1 6.5*: a 6.5 kb full length rat Cat-1 cDNA (Aulak et al., 1996). (iii) *PEPCK*: a 1.1 kb Pst I fragment from the 3′ end of the cDNA for the phosphoenolpyruvate carboxykinase gene (*PEPCK*) (Beale et al., 1985). (iv) *albumin*: a 1.0 kb DNA insert from the pALB cDNA (Groot et al., 1984). (v) *TAT*: the cDNA for the tyrosine aminotransferase gene (Ganns et al., 1994). (vi) *gapdh*: a 1.4 kb glyceraldehyde-3-phosphate dehydrogenase cDNA (Groot et al., 1984), (vii) *c-fos*: a 1.0 kb fragment of the c-fos cDNA (Aulak et al., 1996). The probes which were used for Northern blot analysis were labeled using the random priming kit from Boehringer Mannheim and the specific activity of the probes were 10^8–10^9 cpm/μg DNA.

RNA extraction and Northern blots

Methods described previously were used for RNA analysis (Ausubel, 1993; Maniatis et al., 1982). Frozen liver tissue pieces or tissue culture cells were placed into 4 M guanine thiocyanate buffer (4 M GTC, 0.5% sarcosyl, 25 mM sodium citrate pH 7.0). The samples were immediately homogenized. The homogenate was then loaded onto a cushion of CsCl (5.7 M CsCl, 0.1 mM EDTA pH 7.0) and spun at 175,000 g for 16 h. After centrifugation, the pellet was dissolved in Hepes buffer(10 mM Hepes, 1 mM EDTA, 0.1% SDS, pH 7.5) and precipitated with 2.5 volumes of ethanol and 0.3 M sodium acetate. The precipitate was then dissolved in DEPC-treated water and samples were immediately frozen at −80°C until required.

For Northern blots, 25 μg total RNA was denatured using methyl-mercury (15 mM methyl mercury, 1% SDS, borate buffer, 15% glycerol) and analyzed on a 1% agarose gel. RNA was transferred onto Gene-screen plus and hybridized with the appropriate DNA hybridization probes in hybridization buffer (0.25 M NaCl, 0.25 M sodium phosphate, 1 mM EDTA, 7% SDS, 0.25 mg/ml salmon sperm DNA, 1 mg/ml dry non-fat milk, 50% de ionized formamide) at 42°C. Blots were washed in 0.1% SDS and 1X SSC (SSC consists of 150 mM NaCl and 15 mM sodium citrate). The percentage of SDS in all solutions is weight/volume.

Treatment of cells and animals with hormones

Tissue culture cells

FTO2B cells (Killary et al., 1984) were maintained in DMEM/F12 media supplemented with 5% fetal bovine and 5% bovine serum. The cells were transferred to either media without serum or media without serum containing one or combination of the following components: 50 nM porcine insulin, 1 μM dexamethasone, 10 μg/ml actinomycin D (ActD), 10 μg/ml cycloheximide (cx), or 1 mM puromycin. Cells were incubated for the time indicated in the figure legends. RNA was isolated from the cells and analyzed by Northern blot analysis. The gapdh and albumin patterns of hybridization were used to normalize the loading of the RNA on the Northern blots.

Rats

Male Sprague Dawley rats (50 g) were used for these studies. All experiments started at 10:00 AM. Rats received intraperitoneal injection of insulin (4 U/kg body weight) which was dissolved in a 50% glucose/saline solution. Dexamethasone (1 mg/kg body weight) was injected intraperitoneally in saline. The rats were sacrificed at the indicated time and the livers were used in part for the isolation of nuclei. The remainder was frozen in liquid nitrogen to be used later for RNA isolation.

Nuclear run-off assays

Nuclei were prepared from rat livers as previously described (Aulak et al., 1996). Briefly, fresh liver tissue was homogenized in homogenization buffer (15 mM Hepes, 60 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 5 mM DTT) containing 0.3 M sucrose. The nuclei were then pelleted by centrifugation through a cushion of homogenization buffer and the recovered nuclei were washed, suspended, counted and stored in transcription buffer. Nuclear run-on assays were performed by a detailed method that we described previously (Aulak et al., 1996). The transcription of the *PEPCK* gene was measured as a positive control of genes known to be transcriptionally regulated by hormones in the liver. The vector pBluescript served as the negative hybridization control and genomic DNA was used to normalize the efficiency of the nuclear run off reactions in each sample. Therefore, the visual comparison of intensities of the hybridization signals for Cat-1 in Fig. 1, should be made relative to the corresponding signals for genomic DNA. The rate of transcription of the *Cat-1* and *PEPCK* genes was quantified by using the phosphorimager (Molecular Dynamics). Densitometric analysis of the autoradiograms was also performed using the CS SCAN 5000 densitometer. Different timed autoradiographs were used for quantitation, to ensure that the exposures were within the linear range of the x-ray film. In Fig. 1 we present the autoradiograms and the quantification of the transcription rates as determined by the phosphorimager. The efficiency of transcription of the nuclei was normalized against transcription of total rat genomic DNA. Since transcription of many genes is regulated in the liver, the choice of genomic DNA was more reliable and gave us reproducible data. The fold-induction of transcription over control (0 time point) was estimated as the ratio of the individual DNA autoradiographic signals over the signal of total rat genomic DNA.

Results

Our hypothesis was that expression of Cat-1 is induced in the quiescent liver when cationic amino acid transport is required. The liver is involved in uptake of 50% of amino acids following a meal. Given that insulin is a key mediator of energy balance, we suggested that insulin might be a regulator of *Cat-1* gene expression in response to feeding in physiologic conditions. On the other

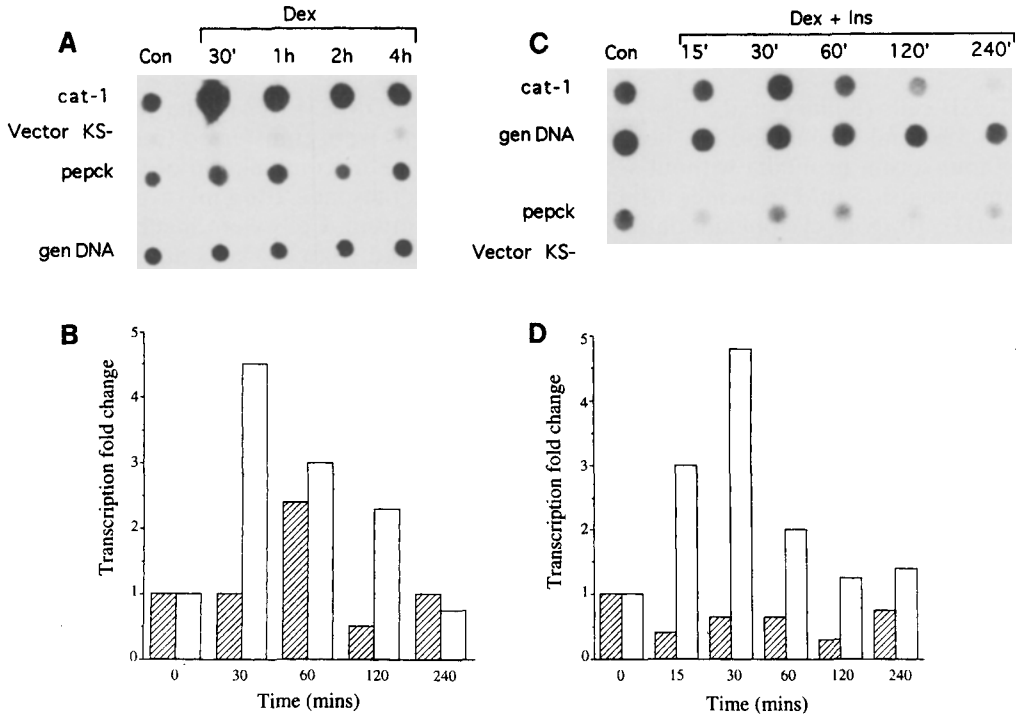

Fig. 1. Run off analysis of liver cell nuclei. Nuclei were prepared from the liver of control rats and rats injected with dexamethasone (**A**) or dexamethasone and insulin (**C**) at the time points indicated in the figure. *In vitro* labeled RNAs were hybridized to DNA immobilized on nitrocellulose paper. Identical nitrocellulose strips containing Cat-1 (6.5 kb cDNA), vector KS-(blue script), PEPCK (1.5 kb rat cDNA) and rat genomic DNA were used for each group (**A** and **C**). The autoradiograms in A and C are representative of one out of three experiments performed. Autoradiograms of different exposure time were used to evaluate the fold change of transcriptional activity, as described in the Methods section. The fold change of transcription has been plotted (**B** and **D**) versus time after injection of dexamethasone (**B**) or dexamethasone and insulin (**D**), for Cat-1 (open bars) and PEPCK (striped bars)

hand, hepatic amino acid uptake of arginine and lysine increases significantly during catabolic states and glucocorticoids play a key role in mediation of the hepatic response. We have investigated the regulation of Cat-1 by glucocorticoids and insulin in the quiescent rat liver.

Glucocorticoids and insulin induce transcription of the Cat-1 gene in the quiescent rat liver

We have shown earlier that the level of Cat-1 mRNA increased in the livers of rats that were fed a high carbohydrate /low protein diet (Wu et al., 1994). Which suggested that insulin is involved in the regulation of hepatic Cat-1 gene expression during nutritional changes of the rats. Furthermore, we have shown that Cat-1 mRNAs accumulate in the liver after injection of glucocorticoids or insulin (Wu et al., 1994). To determine the molecular

mechanism of Cat-1 mRNA induction, we performed nuclear run-off experiments on nuclei isolated from the livers of rats treated with the two hormones.

Treatment of rats with dexamethasone induced transcription of Cat-1 at 30 min (4.6 fold) which returned to control level at 4 hours (Fig. 1, A and B). The pattern of induction of the Cat-1 mRNAs was different than the pattern of induction of transcription. Our earlier studies indicated that expression of the *Cat-1* gene in the rat liver, results in the accumulation of two mRNAs at 7.9 and 3.4 kb, with the concentration of the 7.9 kb mRNA being 5 times higher than the 3.4 kb (Wu et al., 1994). The two mRNAs result from the usage of alternative polyadenylation signals within the 3′ untranslated region of the *Cat-1* gene (Aulak et al., 1996). In our present study, we demonstrate that following treatment of rats with dexamethasone, the 7.9 kb mRNA showed two peaks of accumulation at 30 min and 2 h (Fig. 2A). The 3.4 kb mRNA was also induced, but in a lesser extent than the 7.9 kb (data not shown). In contrast to the two peaks of Cat-1 mRNA accumulation, glucocorticoids caused an expected (Ganss et al., 1994) gradual increase in tyrosine aminotransferase mRNA (Fig. 2A). The level of the albumin mRNA which is not regulated by glucocorticoids was measured to normalize the amount of RNA loaded on the gel (Fig. 2A). The pattern of the hepatic Cat-1 transcriptional activation and mRNA induction following treatment with the hormones, was reproducible in three independent experiments.

The same transcriptional activation and mRNA induction patterns for the *Cat-1* gene were observed following treatment of rats with insulin. The transcription rate of Cat-1 was induced by insulin at 30 min (4.5 fold) and returned to basal level after 4 hr (data not shown). Two peaks of accumulation for the 7.9 kb mRNA were observed at 30 min and 2 hr (Fig. 2B). As expected, the level of gapdh mRNA did not change (Fig. 2B).

As a positive control for the transcriptional regulation of metabolic genes in the liver by glucocorticoids, we have measured the transcription rate for the *PEPCK* gene in the same rat liver nuclei as in Fig. 1A. It is well known that the transcription of the gene for *PEPCK* is induced by glucagon and glucocorticoids (Lamers et al., 1982) and inhibited by insulin (O'Brien et al., 1996). Treatments of rats was performed in the morning when rats are in the fed state. At the fed state the basal level of transcription for the *PEPCK* gene is anticipated to be low (O'Brien et al., 1996; Lamers et al., 1982). Glucocorticoids and glucagon (through cAMP) also stabilize the PEPCK mRNA (Petersen et al., 1989; Nachaliel et al., 1993). As expected, dexamethasone transiently increased transcription of the *PEPCK* gene in 60 min (Fig. 1A and B). In rats treated with insulin ($t_{1/2}$ of insulin in serum is 10–15 min), accumulation of the PEPCK mRNA which has a half life of 20 min *in vivo*, was transiently decreased over the first two hr of treatment (Fig. 2B). In agreement with previous studies (Lamers et al., 1982), this transient decrease was propably due to the negative regulation of the *PEPCK* gene transcription by the heightened serum insulin. The level of the PEPCK mRNA then increased to control levels between 2 and 4 h after insulin injection (Fig. 2B).

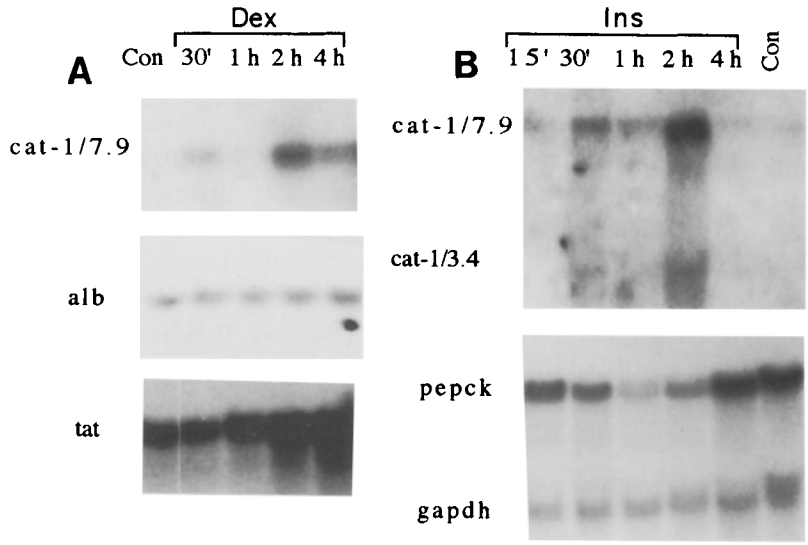

Fig. 2. Northern blot analysis of Cat-1 mRNA from the liver of rats injected with hormones. Total RNA (20 μg), from rats injected with (**A**) dexamethasone for 30 min, 1 h, 2 h and 4 h, was analyzed using the *Cat-1/2.5, alb* and *TAT* hybridization DNA probes. **B** Insulin for 15, 30 min, 1 h, 2 h and 4 h, was analyzed using the *Cat-1/2.5*, *PEPCK* and *gapdh* hybridization DNA probes. Three rats per group were used and the data obtained were similar

In order to determine if there is a synergism of the two hormones on the transcription rate of the *Cat-1* gene, we performed nuclear run-off experiments on nuclei isolated from the livers of rats injected with insulin and glucocorticoids (Fig. 1C and D). When both hormones were injected simultaneously, the transcription rate of the *Cat-1* gene increased 4.8-fold after 30 min and returned to basal level by 4 hours, indicating that there is no synergism. The pattern of Cat-1 mRNA accumulation in the same rat livers was similar to the one observed for the individual hormones (data not shown). Transcription of the *PEPCK* gene in rats treated simultaneously with insulin and dexamethasone, remained low (Fig. 1C and D), due to the inhibitory effect of the injected insulin and the elevated serum insulin level produced by the injection of dexamethasone (Dallman et al., 1993).

The data described above, indicate that glucocorticoids and insulin induce transcription of the *Cat-1* gene. However, we did not observe any synergism of the two hormones on the transcription rate of the *Cat-1* gene.

Induction of expression of the Cat-1 gene by glucocorticoids and insulin is sensitive to inhibitors of transcription and protein synthesis

The factors which are involved in the regulation of expression of the *Cat-1* gene in liver and hepatoma cells are not known. Cat-1 mRNA level is high in hepatoma cells (Wu et al., 1994; Aulak et al., 1996) and y^+ cationic amino acid transport is induced relative to the quiescent liver (White, 1985; White et al.,

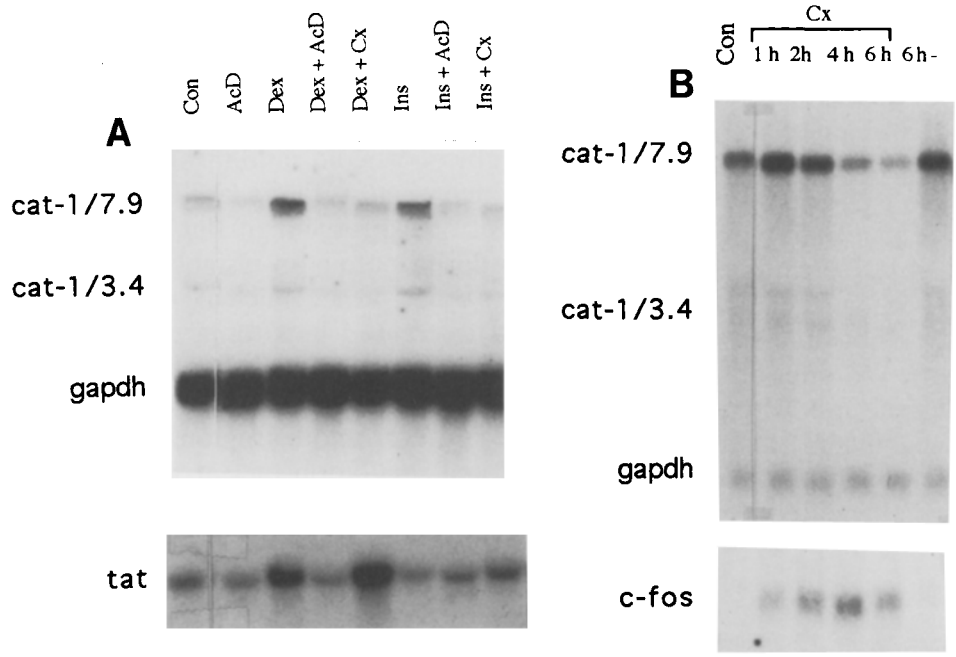

Fig. 3. Effect of ActD and cx on the concentration of Cat-1 mRNAs in rat hepatoma cells following treatment with hormones. Northern blot analysis of total RNA isolated from FTO2B rat hepatoma cells: **A** treated for 2 1/2 hr with dexamethasone or insulin in the presence or not of cx and ActD, in serum free media. The hybridization probes were *Cat-1/2.5*, and *gapdh*. The same blot was used for hybridization with the *TAT* DNA probe. **B** Treated with cx for 1–6 hr, in serum free media, using the *Cat-1/6.5*, *c-fos* and *gapdh* DNA probes. The last lane (6h-) contains RNA from cells maintained in serum free media in the absence of cx

1982). However, the transcription rate of Cat-1 in hepatoma cells is lower than the quiescent liver (Aulak et al., 1996; Hyatt et al., 1997). In order to determine if the induction of the Cat-1 mRNAs by the two hormones depends on protein synthesis and transcription, we examined the effect of ActD and cx on the hormone-induced accumulation of the Cat-1 mRNAs in FTO2B rat hepatoma cells. FTO2B cells is a well differentiated cell line which expresses the liver-specific genes *PEPCK*, albumin and tyrosine aminotransferase. Treatment of cells with ActD or cx, abolished hormone-induced accumulation of the mRNAs, following 21/2 hours of treatment (Fig. 3A). Furthermore, treatment of cells with cx reduced the basal level of Cat-1-mRNA by 70% after six hours (Fig. 3B). However, Cat-1 mRNA levels transiently increased the first hour of treatment with cx. Treatment of cells with another translational inhibitor, puromycin which inhibites protein elongation, decreased the basal level of Cat-1 mRNAs at the same rate as cx (data not shown). In contrast to the decay of Cat-1 mRNA in response to protein synthesis inhibitors, we have observed the expected induction of c-fos mRNA level by these agents (Fig. 3B). Furtheremore, as expected (Ganss et al., 1994), glucocorticoids induced TAT-mRNA level in a manner independent of protein synthesis (Fig. 3A). While reports on the regulation of the *TAT* gene by

insulin are controversial (Ganss et al., 1994), we have observed that insulin blocks the glucocorticoid-induced TAT mRNA level (data not shown). We did not observe any significant change in the basal TAT mRNA level following treatment of FTO2B cells by insulin (Fig. 3A). We conclude that the increase of the Cat-1 mRNA level by glucocorticoids and insulin depends on de novo RNA and protein synthesis, which suggests that a labile factor may be involved in maintaining the basal level of expression of the *Cat-1* gene or Cat-1 mRNA, in hepatoma cells. In order to obtain an insight in the mechanism of *Cat-1* gene-regulation by cx, we performed nuclear run-off experiments, using nuclei from control and cx-treated hepatoma cells (data not shown). We only observed a small decrease of the transcription rate of the *Cat-1* gene following cx treatment of cells for 6 h, which may not be statistically significant (data not shown). Once the promoter region of the gene is characterized, the possible transcriptional regulation of Cat-1 by cx can be further elucidated.

Discussion

The liver plays a central role in the metabolism of amino acids; glucocorticoids and insulin regulate these metabolic processes (Cynober et al., 1995). We have determined in the present study the regulation of expression of the gene for the arginine transporter Cat-1 in liver cells, in response to insulin and glucocorticoids. We propose that expression of Cat-1 in the quiescent liver is subject to hormonal and nutritional regulation by increased transcription of the Cat-1 and subsequent stabilization of the Cat-1 mRNA.

Transport of arginine and lysine through system y^+ is also regulated at the post-translational level by a mechanism called *trans*-stimulation. *Trans*-stimulation involves an increase in arginine transport by the intracellular concentration of cationic amino acids (White et al., 1982). *Trans*-stimulation occurs more readily with Cat-1 than Cat-2, owing to exchange. This mode of regulation of y^+ transport suggests that low levels of the transporter protein can support cationic amino acid transport for metabolic processes. It is therefore possible that transient induction of low level Cat-1 mRNAs, that we observed in this study, are adequate to support arginine transport in exchange for cationic amino acids when it is required. Therefore increased Cat-1 expression may increase the net arginine uptake by a mechanism of exchange with other cationic amino acids.

The molecular factors which regulate hepatic cationic amino acid transport are not known. However, the fact that Cat-1 mRNA and y^+ transport activity are absent in the normal quiescent liver (White et al., 1982; Aulak et al., 1996) and appear after treatment of rats with hormones (Wu et al., 1994), suggests that amino acid transport is regulated at the molecular level. This is also supported by the reports on the regulation of *Cat-1* gene expression and arginine transport by insulin and inflammatory cytokines (Simmons et al., 1996) in cardiac myocytes.

We have shown in this paper that both insulin and glucocorticoids induce the expression of the *Cat-1* gene in the quiescent liver with similar transcrip-

tional/RNA accumulation profiles. Our finding that there is no synergism by the two hormones on the transcription rate of Cat-1, supports our earlier observation of the lack of synergism by the two hormones on the accumulation of the Cat-1 mRNAs in hepatoma cells (Wu et al., 1994). This may indicate that the mechanism of transcriptional regulation of *Cat-1* gene expression by the two hormones may share a common mode of action, or competing molecular sites. For example, if the hormone/receptor complex binds at overlapping DNA sequences within the promoter/enhancer region of the gene, no synergistic action on transcription would occur. Alternatively, regulation may occur through a limiting shared factor for which both mediators of the insulin signal transduction pathway and hormone/receptor complexes, compete for binding. Nevertheless, both hormones increase the transcription rate of the Cat-1 gene 4–5 fold within the first 30 minutes, followed by a decay to basal level after 4–8 hours. Accumulation of the Cat-1 mRNAs occurs in two peaks, a rapid response peak at 30 minutes and a second peak at 2 h. The first peak falls to basal level at 1 h, before the appearance of the second peak at 2 h. This result is surprising since our previous calculation on the half life of the Cat-1 mRNAs in FTO2B cells was 90 minutes for the 7.9 kb and 250 min for the 3.4 kb mRNAs. Given that the first peak of Cat-1 mRNA accumulation decays so rapidly, it suggests a much shorter half life of the induced Cat-1 mRNA in the liver (Aulak et al., 1996). Given that the hepatic transcription rate of the *Cat-1* gene is induced in a single peak and the mRNA accumulation is induced in a double peak, regulation of expression of the *Cat-1* gene must involve both a transcriptional and post-transcriptional component. We have previously shown that in the regenerating liver, the transcription rate of the *Cat-1* gene did not change whereas the level of the Cat-1 mRNAs increased, suggesting a post-transcriptional mechanism of regulation (Aulak et al., 1996). We postulated then, that a labile protein which promotes RNA stabilization was induced resulting in a longer half life of the Cat-1 mRNAs thereby leading to its accumulation. In order to explain the two peaks of mRNA accumulation in response to the hormonal treatments of the quiescent liver, we have put forward a similar hypothesis. In the quiescent liver, the $t_{1/2}$ of the Cat-1 mRNAs is very short due to the absence of a stabilizing factor. The rapid increase in transcription, induced by the hormones, results in the accumulation of the RNAs. Once transcription falls below a threshold value the rate of decay of the Cat-1 mRNA exceeds accumulation resulting in the fall of the first mRNA peak. The second phase of this response elicits the same effect implied by the post-transcriptional mechanism of *Cat-1* gene regulation in the regenerating liver (Aulak et al., 1996). Following hormonal treatment, the stabilizing protein is induced, giving rise to increased half-life of the Cat-1 mRNA resulting in the second peak. The kinetics of the second peak (2 h) are similar to the peak seen with partial hepatectomy (2–4 h) suggesting that Cat-1 mRNA stabilization occurs over a longer time compared with the immediate effect on transcription. An interesting possibility might be that the Cat-1 mRNA may initially (the first hour following transcriptional induction) reside in a cellular pool that makes it accessible to the decay factor, such as free mRNA-ribonucleoprotein com-

plexes and later shifts to a more stable pool, such as bound to polysomes (Ross, 1995).

Overall, suppression of expression of the *Cat-1* gene in the quiescent liver is dominant, since the induction of transcription and mRNA accumulation was only transient. We conclude that the first peak of Cat-1 mRNA at 30 min, is the result of transcriptional activation, but the majority of this RNA is degraded. The second peak at 2 hours is the result of mRNA stabilization. Another example of two peaks of mRNA accumulation in the adult rat liver in response to hormonal and dietetic changes is the regulation of expression of the *PEPCK* gene (Lamers et al., 1982). It has been shown that the PEPCK mRNA which is synthesized in the first 60 min of carbohydrate-refeeding following 24 hr of starvation is immediately degraded (Lamers et al., 1982). Alteration in the stability of hnRNA in the nucleus is also known to occur in the quiescent liver (Goumaz et al., 1994).

The mechanism of the transcriptional and post-transcriptional regulation of the *Cat-1* gene is not known, given that the promoter region and the full length cDNA (Aulak et al., 1996) have not been studied. However, we have demonstrated in this study, using rat hepatoma cells, that the induction of expression of the *Cat-1* gene by glucocorticoids and insulin is inhibited by the protein synthesis inhibitors cx and puromycin and by the inhibitor of transcription ActD. Furthermore, treatment of cells with cx reduced the Cat-1 mRNA level by 70% after six hours, when the transcription rate of the gene did not decrease significantly. These data support the hypothesis that a labile protein factor is required for maintainance of steady state Cat-1 mRNA in hepatoma cells. Two possible models can be considered to explain these data. Treatment of cells with cx either changes the steady state level of the Cat-1 mRNAs by increasing decay of the transcribed mRNA, or it has an effect directly on the hormone-induced transcription of the Cat-1 gene. The first model suggests that a labile protein may be involved in mRNA turnover. The second model suggests that transcription and hormonal regulation of the *Cat-1* gene is supported by a labile transcription factor. With regard to the second model, the labile transcription factor, may be required for the hormone-receptor complex to induce transcription of the gene. An example of this, is the inhibition of the glucocorticoid-induced expression of the acute phase α-1 acid glycoprotein by inhibitors of protein synthesis (Klein et al., 1987). Sequences within the promoter of the α-1 acid glycoprotein gene have been shown to mediate cx-inhibition of steroid induced expression (Klein et al., 1987). In contrast to our data, induction of gene expression by glucocorticoids, such as genes linked to the MMTV promoter, has been mainly shown to be independent of protein synthesis. Given that both protein synthesis inhibitors cx and puromycin negatively regulate Cat-1 mRNA accumulation in hepatoma cells, we suggest that labile factors are involved in *Cat-1* gene expression and mRNA turn over. However, given that in hepatoma cells treated with cx, we observed a small decrease in the transcription rate of the gene and decrease in mRNA accumulation by 70% in 6 h, regulation of *Cat-1* gene expression in hepatoma cells appears to be primarily at the post-transcriptional level by mediating mRNA turn over. This is partly in agree-

ment with our earlier studies, in which we demonstrated that Cat-1 is a delayed early growth response gene in the regenerating rat liver and its expression depends on protein synthesis (Aulak et al., 1996). Given that FTO2B cells have characteristics of regenerating liver cells (Killary et al., 1984), regulation of Cat-1 may be similar to the regenerating liver. In contrast to the regenerating liver(Aulak, 1996), where regulation of expression of the Cat-1 gene is post-transcriptional, in the quiescent liver Cat-1 is also regulated at the transcriptional level. The mechanism of this regulation has to be determined. Our working hypothesis is that a trans-acting factor stabilizes the Cat-1 mRNA in the quiescent and regenerating liver, as well as in hepatoma cells. It is likely that this factor is regulated and preferbly induced in transformed cells. There is extensive literature on positive and negative regulation of gene transcription by glucocorticoids and insulin (O'Brien et al., 1996). As with the transcriptional regulation of gene expression, insulin and glucocorticoids both increase and decrease the stability of specific mRNAs in the same cell (O'Brien et al., 1996). For example, insulin inhibits transcription of the albumin gene in hepatoma cells and represses the albumin mRNA levels by an unknown post-transcriptional mechanism (O'Brien et al., 1996). However, studies on the mechanism(s) of post-transcriptional regulation of gene expression lag behind the overwhelming literature of transcriptional regulation.

The regulation of *Cat-1* gene-expression is similar to the regulation of System A amino acid transport activity (Laine et al., 1987). System A mediated amino acid transport is induced by insulin, diabetes, glucocorticoids and amino acid starvation (McGivan et al., 1994). Several studies on system A and other amino acid transport systems (Laine et al., 1987; Felipe et al., 1992; Plakidou-Dymock et al., 1993) support regulation of amino acid transport at the molecular level. Given that the cDNA(s) for System A mediated amino acid transport have not been cloned, direct studies at the molecular level are not available.

The physiologic significance of the complex regulation of expression of the transporter genes in the liver, reflects the careful control of arginine transport across the cell membrane of liver cells (Cheeseman, 1991). If this process is not tightly controlled, the transport of arginine by the hepatocytes, followed by arginine catabolism through the urea cycle, could rapidly deplete the plasma pool of arginine, with serious metabolic consequences (Cynober, 1995). In addition, under conditions where the urea cycle enzyme arginase is down regulated, such as in adrenalectomized rats (Viru et al., 1994), induction of expression of Cat-1, may allow the hepatocytes to release arginine and ornithine into the plasma circulation.

The transient induction of hepatic *Cat-1* gene expression by insulin in the quiescent liver, may be important in providing cationic amino acids for metabolic pathways. In hepatocytes, insulin regulates a variety of metabolic functions including transmembrane amino acid transport and protein turn over (Cynober, 1995). Induction of Cat-1 by glucocorticoids may be important in enhancing the hepatic amino acid transport of arginine and lysine during catabolic states (Inoue et al., 1995; Fisher et al., 1995; Pacitti et al., 1992).

Furthermore, glucocorticoids also induce the transcription and translation of acute phase proteins (Baumann et al., 1989) suggesting that there is coordinate action of the hormone on the expression of the amino acid transporter genes to support protein synthesis. Additionally, the induction of expression of the hepatic arginase by glucocorticoids may be linked to induction of Cat-1, which will increase arginine supply when its catabolism is accelerated in the hepatocytes.

Although, in the present study, we have examined the effect of glucocorticoids and insulin on the regulation of expression of the *Cat-1* gene, the expression of this gene is also regulated by a number of other factors, such as cell density (Wu et al., 1994), cell growth (Laine et al., 1996), phorbol esters (Yoshimoto et al., 1992), inflammatory cytokines (Simmons et al., 1996), platelet derived growth factor (Durante et al., 1996), angiotensin II (Low et al., 1995) and amino acid starvation (Hyatt, 1997). In cardiac smooth muscle cells, insulin and cytokines synergistically act to increase expression of the *Cat-1* gene (Simmons et al., 1996). Pacitti et al. also reported that the increased hepatic amino acid transport elicited by inflamatory cytokines is partially mediated by glucocorticoids (Pacitti et al., 1992). This complex regulation may be needed to control cationic amino acid transport according to the needs and function of different tissues. In support of this complex regulation is the cloning of the promoter for the transporter Cat-2/2a gene, which indicated that multiple promoters and alternative RNA splicing regulate the expression of this gene (Finley et al., 1995; Stevens et al., 1996).

We suggest, that transport of cationic amino acids across the plasma membrane of liver cells is carefully controlled through the transcriptional and post-transcriptional regulation of the *Cat-1* gene. In tumor cells cationic amino acid transport is controlled by the y^+ system and in some tumor cells by the $B^{0,+}$ and $b^{0,+}$ systems (Laine et al., 1996). Given that expression of the *Cat-1* gene is highly induced in tumor cells (Yoshimoto et al., 1992), it should be a significant transporter of cationic amino acids in the rapidly growing tumors for protein and polyamine biosynthesis. Understanding the mechanism of regulation of expression of the cationic amino acid transporter genes in quiescent and dividing cells will further assist the design of therapies to control tumor growth.

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Authors' address: Maria Hatzoglou, PhD, Department of Nutrition, Case Western Reserve University School of Medicine, Cleveland, OH 44106, U.S.A.,
Fax (216)368-6644, Email mxh8@po.cwru.edu

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