

Changes in levels of argininosuccinate lyase mRNA during induction by glucagon and cyclic AMP in cultured foetal-rat hepatocytes

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During the perinatal period, the activity of the urea-cycle enzyme argininosuccinate lyase (ASL) is regulated by glucocorticoids, glucagon and insulin. In this study, the effects of glucagon and cyclic AMP (cAMP) analogues were examined on the synthesis of ASL and on the level of its corresponding mRNA in cultured foetal hepatocytes. Northern-blot analysis revealed that these agents only gave a transient induction of ASL mRNA amount, which reached a peak at 6 h and declined thereafter. This induction preceded the increase in enzyme activity and amount which could be observed for 2 or 3 days of culture. Stimulation

of ASL mRNA accumulation by a combination of cAMP analogues and dexamethasone was additive, indicating that glucocorticoids and cAMP are both necessary to promote hepatocyte differentiation and that inductions could occur via independent pathways. Induction by cAMP analogues could be abolished by actinomycin D, suggesting a control mechanism at the transcriptional level. Puromycin was without effect on ASL mRNA induction by cAMP, indicating that no ongoing protein synthesis was required in the stimulation process.

INTRODUCTION

The urea-cycle enzyme argininosuccinate lyase (ASL; EC 4.3.2.1) is under multi-hormonal control during development and represents a convenient model for study of regulation of gene expression in foetal mammalian hepatocytes. The enzyme protein and its corresponding mRNA first appear in foetal-rat liver about 6 days before birth [1]. Their levels rise slowly as term approaches and then rise rapidly during the first postnatal day [2–4]. They reach the adult level about 3 weeks after birth [4]. The mechanisms that are responsible for this perinatal development have not yet been completely clarified. Glucocorticoids and glucagon have been implicated in the developmental regulation of this enzyme activity [3–7] as well as its maintenance in the adult rat liver [8–11]. Glucocorticoids are thought to induce ASL by activating transcription [12,1], and we asked whether glucagon acts through a similar mechanism. In adult rat liver, glucagon was shown to produce an increase in enzyme activity [9,10] and in the amount of ASL mRNA, *in vivo* [12] as well as *in vitro* [13]. Our previous results obtained during the foetal period showed that glucagon or dibutyryl cyclic AMP (Bt₂cAMP) could cause premature induction of the enzyme activity when injected into foetal rats [7,14] or when added to cultured foetal hepatocytes [15,16]. This system allows maintenance of measurable levels of ASL mRNA in control medium for a few days [1] and thus offers a suitable model to study the developmental regulation of this gene expression.

To establish whether the increase in ASL activity after glucagon treatment is regulated at a transcriptional or translational level, an immunochemical test (e.l.i.s.a.) was used to quantify the amount of ASL [4], and we measured hybridizable ASL mRNA using a cDNA probe [17] in cultured foetal hepatocytes maintained under various hormonal conditions. Our results lead to the conclusion that glucagon, acting via cAMP, induces ASL

activity *in vitro* by promoting a transient accumulation of specific mRNA.

MATERIALS AND METHODS

Materials

Animals

Adult female rats of the Wistar strain, kept under standard conditions, were mated overnight and recognized as pregnant by a vaginal smear on the next morning. Parturition was at 21.5 days. Foetuses were obtained by Caesarean section on the appropriate day of gestation.

Chemicals

Glucagon was from Novo (Bagsvaerd, Denmark). Argininosuccinate, dexamethasone, N⁶,O²-dibutyryl cAMP (Bt₂cAMP) and puromycin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 8-(4-Chlorophenylthio) cAMP (CPT-cAMP) was from Boehringer (Mannheim, Germany), and actinomycin D was from Calbiochem (San Diego, CA, U.S.A.). The culture medium was a mixture of Eagle's Minimal Essential Medium and Medium 199 with Hanks' salts, deficient in arginine, obtained from Eurobio (Paris, France). The materials used for cell isolation and culture were as reported previously [15]. Guanidinium thiocyanate was purchased from Fluka A.G. (Basel, Switzerland). Hybond N membranes, multiprime DNA-labelling system and [α -³²P]dCTP were from Amersham Corp. The plasmid pAlr4 [17] was a gift from Dr. M. Mori (Kumamoto University, Japan). The chicken β -actin probe and the rat phosphoenolpyruvate carboxykinase (PEPCK) probe (pPCK-10), respectively developed by Cleveland et al. [18] and by Yoo-Waren et al. [19], were used as internal controls. X-ray film (XAR-5) was from Eastman Kodak (Rochester, NY, U.S.A.).

Cronex Quanta III intensifying screens were obtained from Du Pont de Nemours (Wilmington, DE, U.S.A.).

Methods

Culture of foetal rat hepatocytes

Hepatocytes from 18.5-day foetuses were cultured by the method of Leffert and Paul [20] with some modifications [15]. The cells were suspended in the culture growth medium containing 10% charcoal-treated foetal-calf serum and cultured in 75 cm² Falcon tissue-culture flasks (Falcon, Oxnard, CA, U.S.A.) at 37 °C under 5% CO₂ in air. After an attachment period of 4 h and every 24 h thereafter, the medium was replaced by fresh medium with 5% foetal-calf serum. In all the experiments presented here, foetal hepatocytes were plated on uncoated plastic flasks and allowed to adapt to culture conditions for 24 h before addition of hormones or cyclic nucleotide analogues to the medium. At times indicated in the Figure legends, cells were harvested and homogenized after 2 or 3 days in culture.

Enzymic and immunochemical tests

Foetal hepatocytes were homogenized in 0.1% cetyltrimethylammonium bromide. Assay of ASL, based on the colorimetric determination of urea, was performed by the procedure of Tomlinson and Westall [21]. The enzyme activity was expressed in μmol of urea formed/h (units) per mg of protein. Quantification of ASL in foetal hepatocytes was performed by a 'sandwich non-competitive e.l.i.s.a.' developed in this laboratory [4]. Immobilized purified IgG was incubated with purified ASL as standard or with hepatocyte extracts for tests. After washing, the immobilized antibody-antigen complex was incubated with an excess of peroxidase-labelled antibody, which bound to the remaining antigenic sites. After incubation with peroxidase substrate, the reaction product was measured at 405 nm. The amount of ASL was given as μg of protein. The protein content of homogenates was determined by the method of Lowry et al. [22], with BSA as standard. Significance was determined by Student's *t* test.

Extraction and analysis of cellular RNA

Isolation of total cellular RNA was performed with hepatocytes from four pooled flasks. The monolayers were rapidly washed with phosphate-buffered saline, pH 7.5, and scraped with a rubber spatula. Hepatocytes were collected by centrifugation for 5 min at 700 *g*, frozen in liquid nitrogen and stored at -70 °C before homogenization. Total cellular RNA was extracted by the guanidinium thiocyanate procedure of Cathala et al. [23]. The RNA fractions were precipitated with ethanol and stored at -70 °C. The purity of total RNA was estimated by spectrophotometry (A_{260}/A_{280}) and its integrity was verified by electrophoresis in agarose gels with detection by ethidium bromide u.v. fluorescence. RNA concentrations were determined from the A_{260} . For Northern blotting analysis, 25 μg of total RNA was denatured in formaldehyde and electrophoresed on a 1.2%-formaldehyde/agarose gel [24], then blotted overnight on to nylon sheets. Procedures for pre-hybridization, hybridization and post-hybridization washings were performed in accordance with the instructions of the membrane manufacturer. Radioactive labelling of the cDNA probes was done with [α -³²P]dCTP, with random oligonucleotides as primers. Hybridizations were

performed overnight at 42 °C and the nylon sheets were finally exposed to Kodak XR-5 film at -70 °C for periods in the range 6-48 h, by using intensifying screens. Several different exposures were obtained for each blot to optimize the subsequent analyses of the autoradiograms. The intensity of the bands observed on the autoradiograms was quantified by scanning the film with a Shimadzu (CS-930) densitometer.

RESULTS AND DISCUSSION

Effects of glucagon and cyclic nucleotide analogues on ASL activity and amount

Hepatocytes from 18.5-day foetuses were cultured for 24 h in control medium; then 0.1 μM glucagon, 0.5 mM Bt₂cAMP or 50 μM CPT-cAMP was added for an additional 24 h. The ASL activity and amount of immunoreactive protein were first determined and then compared with amounts of specific mRNA. ASL activity was previously shown to attain maximal levels about 20 h after glucagon addition [16], where it was increased about 2-fold over the control values. Table 1 shows that this induced activity was linked to a similar increase in the amount of immunoreactive protein, as measured by the e.l.i.s.a. method. When foetal hepatocytes were exposed to glucagon, Bt₂cAMP or CPT-cAMP and dexamethasone together, the ASL activity and amount increased additively, suggesting that the two agents acted via independent pathways.

The good correlation between both parameters (activity and amount) leads to the conclusion that the rise in ASL activity measured after hormonal treatments was due to enzyme synthesis.

Effects of glucagon and Bt₂cAMP on ASL mRNA

To understand better the process underlying this cAMP-evoked induction of ASL, we examined the accumulation of specific mRNA in such treated hepatocytes. The effects of individual compounds on ASL mRNA levels were first studied at 48 h of culture by Northern-blot analysis, and a representative experiment is shown in Figure 1. Although dexamethasone produced a marked increase in ASL mRNA, glucagon (Figure

Table 1 Effects of glucagon, Bt₂cAMP, CPT-cAMP and dexamethasone on ASL activity and amount in cultured foetal hepatocytes

Hepatocytes from 18.5-day foetuses were cultured for 24 h in control medium, and then for another 24 h with the different indicated compounds. Cells were harvested for enzyme and immunochemical assays. Values are means \pm S.E.M. for the numbers of separate experiments indicated in parentheses: ^a significantly different from controls; ^b significantly different from identical conditions without dexamethasone.

| Treatment | ASL activity (unit/mg of protein) | ASL amount (μg /mg of protein) |
|--------------------------------------|------------------------------------|--|
| Control cells | 0.150 \pm 0.006 (5) | 1.496 \pm 0.124 (5) |
| Dexamethasone (1 μM) | 0.394 \pm 0.024 (5) ^a | 3.861 \pm 0.254 (5) ^a |
| Glucagon (0.1 μM) | 0.266 \pm 0.035 (5) ^a | 2.342 \pm 0.214 (5) ^a |
| Bt ₂ cAMP (0.5 mM) | 0.291 \pm 0.020 (3) ^a | 2.462 \pm 0.154 (3) ^a |
| CPT-cAMP (50 μM) | 0.300 \pm 0.025 (4) ^a | 3.331 \pm 0.416 (4) ^a |
| Dexamethasone + glucagon | 0.641 \pm 0.095 (4) ^b | 5.803 \pm 0.450 (4) ^b |
| Dexamethasone + Bt ₂ cAMP | 0.601 \pm 0.066 (3) ^b | 5.740 \pm 0.600 (3) ^b |
| Dexamethasone + CPT-cAMP | 0.620 \pm 0.029 (4) ^b | 5.856 \pm 0.871 (4) ^b |

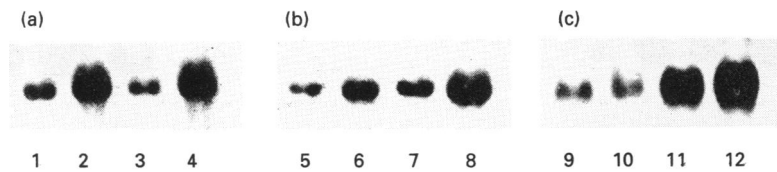


Figure 1 Action of glucagon and Bt₂cAMP on the levels of ASL mRNA in foetal hepatocytes

Hepatocytes from 18.5-day foetuses were maintained in primary culture for 24 h in control medium; then 0.1 μ M glucagon or 0.5 mM Bt₂cAMP was added with or without 1 μ M dexamethasone. Cells were harvested and total RNA was extracted. Samples (25 μ g) of total RNA were subjected to Northern-blot analysis as described in the Materials and methods section. The Figure shows the autoradiograms obtained after hybridizing the RNAs blotted on the nylon sheet with the α -³²P-labelled probes for ASL (a and b) and PEPCK mRNA (c). Lanes 1, 5 and 9, control untreated cells; lanes 2, 6 and 10, dexamethasone; lanes 3 and 11, glucagon; lanes 4 and 12, both glucagon and dexamethasone; lane 7, Bt₂cAMP; lane 8, both Bt₂cAMP and dexamethasone. These results have been reproduced in three independent experiments.

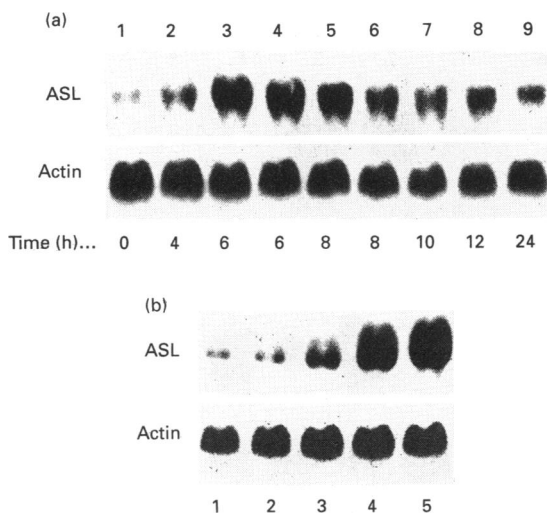


Figure 2 Time course and dose-dependent induction of ASL mRNA by Bt₂cAMP

Hepatocytes from 18.5-day foetuses were cultured for 24 h in control medium; then either 0.5 mM Bt₂cAMP was added and left for the indicated times (a) or Bt₂cAMP was added in various concentrations for 6 h (b). Total RNAs (25 μ g/lane) were electrophoresed, blotted and probed with the ASL cDNA, and then with the β -actin probe. (a) Lane 1, control cells ($t = 0$ h); lanes 2–9, 0.5 mM Bt₂cAMP. (b) Lane 1, control cells; lane 2, 5 μ M Bt₂cAMP; lane 3, 50 μ M Bt₂cAMP; lane 4, 0.5 mM Bt₂cAMP; lane 5, 5 mM Bt₂cAMP.

1a, lanes 3 and 4) was not able to cause a significant increase in ASL mRNA amount 24 h after its addition, in either the presence or the absence of the steroid. Under the same conditions, Bt₂cAMP only produced a marginal increase in ASL mRNA (Figure 1b, lanes 7 and 8). This lack of response to relatively long-term treatments with glucagon or Bt₂cAMP was reproduced in multiple experiments at 24, 48 and 72 h of culture (results not shown). As controls, the same RNA samples were re-hybridized with the PEPCK cDNA, and results (Figure 1c, lanes 11 and 12) demonstrated that glucagon was quite effective in stimulating PEPCK mRNA accumulation 24 h after its addition. This marked contrast with the effects on ASL amount and activity led us to explore whether glucagon or Bt₂cAMP might have an effect on the ASL mRNAs at incubation times different from those used in the preceding experiments. Foetal hepatocytes were incubated in the presence of these compounds for various time periods extending from 1 to 24 h, and the time course of ASL mRNA response to Bt₂cAMP is shown in Figure 2(a). The cyclic

nucleotide analogue was added 24 h after plating and left for the times indicated. In this case an inducing effect was seen at 4 h, and maximal mRNA levels were attained by 6 h, where a nearly 3-fold increase over control levels could be measured. Thereafter the mRNA amount decreased significantly, approaching the initial values by 24 h. This induction was concentration-dependent, and the maximal response of ASL mRNA occurred at 0.5 mM Bt₂cAMP (Figure 2b). The same membranes were also hybridized with the β -actin cDNA, and the results demonstrated that the cyclic nucleotide did not affect the amount of β -actin mRNA and confirmed that the RNA loading was constant. The time course of the ASL mRNA response differed somewhat from that of the ASL activity [16] and amount, which remained high at 24 h (Table 1) even though the mRNA level had returned to basal. Although the quantitative measures precluded precise comparisons between mRNA and protein amounts, this transient increase in ASL mRNA was consistent with the increased level of the protein that was detected 24 h after addition of the inducer. The fact that the increase in enzyme amount and activity lagged behind the increase in mRNA levels might be explained by the different half-life of the protein ASL and its corresponding mRNA. The half-life of the urea-cycle enzymes has been proposed to be in the range of a few days [25], and our results on mRNA stability suggest that the half-life of ASL mRNA can be evaluated as hours (S. Renouf, A. Fairand and A. Husson, unpublished work). Another possibility to explain the different time course of response is that a translational mechanism might control the ASL amount and prevent short-term variations of mRNA. This phenomenon was already observed at birth, when ASL mRNA accumulated sharply in a few hours, whereas the ASL protein accumulated only after a lag time [4]. Such a rapid rise and fall in specific mRNA levels were also observed for tyrosine aminotransferase in cAMP-treated rat foetuses [26].

To understand better the mechanism of the transitory nature of the cAMP-evoked induction of ASL mRNA, we examined the effects of addition and re-addition of Bt₂cAMP to cultured foetal hepatocytes. Fresh Bt₂cAMP re-added at 6 h, when the induction had reached a peak, failed to evoke a new induction of ASL mRNA (Figure 3), whose level decreased similarly as in the preceding experiment (Figure 2a). Similar results were obtained with 50 μ M Bt₂cAMP (results not shown). This suggests that the cyclic nucleotide was still in a sufficient amount in the medium 6 h after its addition and might reveal a desensitization of the cells towards stimulation by Bt₂cAMP [27].

This time course of induction by Bt₂cAMP was different from that obtained from experiments with dexamethasone, where a longer lag period and slower induction kinetics were observed [1]. In particular, the mRNA amount in foetal hepatocytes

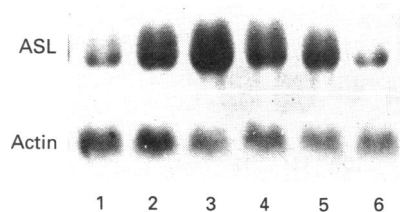


Figure 3 Effects of two additions of Bt_2cAMP on ASL mRNA levels

Hepatocytes from 18.5-day foetuses were cultured in control medium for 24 h (lane 1), then 0.5 mM Bt_2cAMP was added for 6 h and added again at 6 h. Hepatocytes were harvested for RNA preparation at 4 h (lane 2) and 6 h (lane 3) after the first addition, and at 4 h (lane 4), 8 h (lane 5) and 20 h (lane 6) after the second addition. Total RNAs (25 μ g/lane) were electrophoresed, blotted and probed with the ASL cDNA, and then with the β -actin probe.

incubated with dexamethasone remained at a high level for at least 48 h. It was previously shown in Table 1 that the effects of cAMP and glucocorticoids on ASL activity were additive, suggesting that they affect different steps in the synthesis of ASL. We have therefore analysed the combined effects of dexamethasone and Bt_2cAMP at the level of ASL mRNA. At 6 h of incubation, the combined effects of maximal doses of both compounds were approximately additive (Figure 4), since the cyclic nucleotide analogue alone caused about a 2-fold stimulation of ASL mRNA accumulation and a 4-fold one in cells simultaneously incubated with dexamethasone, as evidenced by scanning the autoradiogram of Figure 4. These data indicate that glucocorticoids and cAMP are both necessary to promote differentiation of foetal hepatocytes, and give further support to the idea that the two inducers act by independent mechanisms to elevate the level of this mRNA.

Effects of actinomycin D and puromycin on ASL mRNA levels

The regulation of the synthesis of ASL by cAMP could occur at the level of transcription, or might be the consequence of altered stability of the specific mRNAs. To gain better understanding between these alternatives, we used a RNA-synthesis inhibitor, actinomycin D, and CPT-cAMP (Figure 5a). As expected, in the presence of 50 μ M of this potent cyclic derivative, the amount of ASL mRNA increased rapidly, an increase being apparent within 2 h and reaching a maximum at 6 h. This short-lived increase in mRNA was completely inhibited by the simultaneous addition of actinomycin D, suggesting that the cyclic nucleotide acted at the transcriptional level. Final proof of this transcriptional event might be brought by assays of transcription *in vitro*, although run-on assays were shown to be inconclusive for the ASL gene in isolated nuclei from adult liver [28,29]. Work is in progress to use the run-on assay with nuclei from foetal cells. In contrast, the addition of 20 μ M puromycin, a concentration that usually decreased protein synthesis, had very little effect on the induction of ASL mRNA in Bt_2cAMP -treated cells (Figure 5b). This result suggests that the synthesis of intermediary protein(s) by foetal hepatocytes was not an absolute requirement for increased ASL mRNA synthesis under these culture conditions.

Our results are in general agreement with those obtained from cultured adult hepatocytes [13] and emphasize that sensitivity to glucagon is acquired early in development. In foetal liver, cAMP was shown to induce a variety of liver-specific genes, such as tyrosine aminotransferase or PEPCK [26,30], and our results are also in agreement with more general findings establishing that

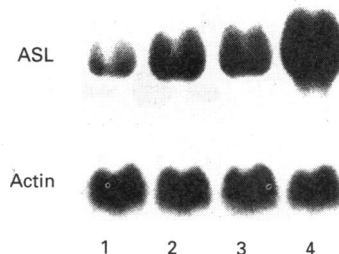


Figure 4 Effects of dexamethasone and Bt_2cAMP on ASL mRNA levels

Hepatocytes from 18.5-day foetuses were cultured for 24 h in control medium; then they were exposed for 6 h to 1 μ M dexamethasone (lane 2) or 0.5 mM Bt_2cAMP (lane 3), alone or in combination (lane 4), or in the absence of either inducing agent (lane 1). Total RNA was isolated from hepatocytes, and 25 μ g samples were subjected to Northern-blot analysis. The same blot was rehybridized with the β -actin probe. This result was obtained in two other experiments.

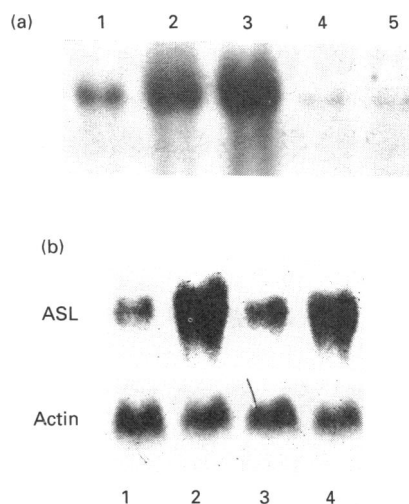


Figure 5 Effects of actinomycin D and puromycin on the induction of ASL mRNA by cAMP analogues in foetal hepatocytes

(a) At 24 h of culture, 18.5-day-foetal hepatocytes were exposed to 50 μ M CPT-cAMP for the times indicated. Lane 1, control cells at $t = 0$ h; lanes 2 and 3, CPT-cAMP for 2 h and 6 h respectively; lane 4, actinomycin D (0.2 μ g/ml) for 6 h; lane 5, both CPT-cAMP and actinomycin D for 6 h. (b) For this, 18.5-day-foetal hepatocytes were exposed to 0.5 mM Bt_2cAMP for 6 h, with (lane 4) or without 20 μ M puromycin (lane 2). Lane 1, control cells; lane 3, puromycin added alone. Samples (25 μ g) of total RNA were applied on each lane. The same membrane was rehybridized with the β -actin probe.

cAMP promotes foetal hepatocyte differentiation *in vitro* [31] at the level of mRNA synthesis. Glucagon has been shown to regulate gene expression by different mechanisms, and some studies have shown the existence of cAMP-responsive elements on specific genes, such as those for PEPCK [32], tyrosine aminotransferase [33], tyrosine hydroxylase [34] and arginase [35]. The ASL gene has been analysed [36] and, although a part of the 5' flanking region has been sequenced [37], functional cAMP-responsive elements have not yet been described, and we do not know whether any sequence elements are actually involved in the response of this gene. Whatever the required mechanism in the cAMP regulation of the ASL gene, our study shows that this mechanism is already functional during foetal life.

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