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Gene-Specific Acquisition of Hormonal Responsiveness in Rat Liver During Development

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Cloned cDNAs were used in hybridization analyses to assess hormonal responsiveness of two similarly regulated genes in livers of late-term fetal rats. Transcription of the tyrosine aminotransferase gene and of gene 33 (Lee et al.: *J Biol Chem 260*:16433–16438, 1985) is enhanced by glucocorticoids and by each of the usually antagonistic hormonal agents, insulin and cAMP, in adult liver, and that of both genes is developmentally activated at or just prior to birth. The mRNA of gene 33 was found to be significantly increased by each of the hormonal regulators in livers of fetuses treated in utero. Expression of the nearly silent aminotransferase gene in fetal liver was appreciably increased by cAMP but was refractory to control by either glucocorticoids or insulin; capacity of this gene to respond to insulin was not realized until several days postpartum. The data indicate specificity in the developmental acquisition of the capacity of individual genes to respond to hormonal regulators.

Key words: hepatocyte differentiation, gene expression, hormonal control, glucocorticoids, insulin, cyclic AMP, tyrosine aminotransferase

Genes whose expression is sensitive to hormonal regulation in the adult fully differentiated liver are not necessarily responsive to controlling hormones in fetal liver. A notable case in point is the gene encoding the liver enzyme tyrosine aminotransferase (L-tyrosine: 2-oxoglutarate aminotransferase, E.C. 2.5.1.5), which is inducible in adult liver by glucocorticoids [1] as well as by each of the classical antagonists insulin and glucagon [2], the latter acting via cAMP [3]. Earlier studies, in which expression was assessed in terms of the enzyme or of the mRNA assayed by translation indicated that tyrosine aminotransferase (TAT) expression is low but detectable in late-term fetal liver, wherein it is somewhat responsive to induction by cAMP but not by either glucocorticoids or insulin [4–6]. In the current work we have used cloned cDNAs as hybridization

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probes to assess hormonal responsiveness of TAT during development in a more definitive fashion and also to compare to another gene that is similarly regulated by these hormones in adult liver. The mRNA of this latter gene, provisionally termed gene 33 pending identification of the protein product, is increased in adult liver by glucocorticoids, insulin, or cAMP in a fashion that is virtually identical, both quantitatively and kinetically, to induction of TAT mRNA by these agents [7]. Here we have asked if this similarity in regulation extends to the attainment of hormonal responsiveness during development.

MATERIALS AND METHODS

Animals and Treatments

CD/CR rats from Charles River Breeding Laboratories were bred and pregnancies were timed as described before [6]; gestational ages of fetuses are accurate to ± 12 hr, and birth is usually on the 22nd day of gestation. Fetal injections were given early on day 20 or 21 of gestation to individual fetuses of anesthetized, laparotomized dams; intraperitoneal injections of 100 μl contained either 100 μg hydrocortisone or 200 μg 8-Br-cAMP or 50 milliunits of insulin or solvent 0.15 M NaCl (littermate or age-matched controls). After treatment the maternal abdomen was sutured closed; at appropriate times the dams were again anesthetized; the fetuses were taken and killed by decapitation, and their livers removed for analysis. Each analysis was made on pooled livers of at least six similarly treated fetuses.

Poly(A)-RNA; cDNA Probes

Postmitochondrial supernatants largely devoid of membrane-bound polysomes were prepared by the centrifugation regimen described previously [8], and poly(A)-RNAs isolated by Mg^{2+} precipitation and oligo(dT)-cellulose chromatography as outlined by Palmiter [9]. The TAT-specific probe pc TAT-3 was generously provided by Dr. Gunther Schutz [10]; that for gene 33 was developed in this laboratory [7]. Both cDNAs were excised from their respective plasmids, purified electrophoretically, and labeled by nick translation to specific activities of $0.5–1.5 \times 10^8$ cpm/μg.

Hybridizations

cDNAs labeled with ^{32}P ($5–7 \times 10^5$ cpm/ml of hybridization solution) were used in Northern blot hybridizations as described [7], except that posthybridization washes were at 65°C. For blot analyses of samples from fetal liver 5 μg of poly(A)-RNA was applied to gels; samples from adult liver contained 1 μg of poly(A)-RNA. For dot hybridization varying amounts of poly(A)-RNAs were bound to nitrocellulose sheets by using the Bethesda Research Laboratory hybridot manifold, and hybridizations were carried out as described by Thomas [11] with ^{32}P-labeled cDNAs as in the blot analyses. Northern blot and dot hybridization data presented are representative of the results of two or more replicate experiments.

RESULTS

Hormonal Control in Adult Liver

To enable comparisons with hormone responses in fetal liver, we have repeated previous experiments indicating that both TAT and gene 33 mRNAs are increased in livers of adrenalectomized adult rats following treatment with either hydrocortisone or

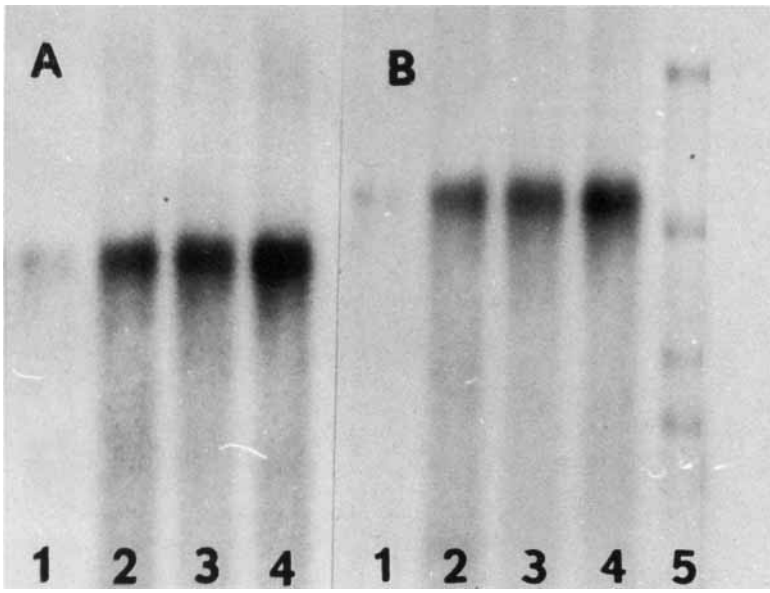

Fig. 1. Northern blot analyses of tyrosine aminotransferase (TAT) and gene 33 mRNAs in adult rat liver after hormonal treatments. Adrenalectomized rats were killed 3 hr after being given hydrocortisone (2.5 mg/100 g) or 1 hr after 8-Br-cAMP (5 mg/100 g) or insulin (1 unit/100 g). Cytoplasmic poly(A)-RNAs (1 μg) were subjected to electrophoresis and blot analysis. **A:** Blot hybridized to the ^{32}P-labeled cDNA of pcTAT-3. Lanes: 1, RNA from liver of untreated control; 2, after insulin treatment; 3, after cAMP treatment; 4, after hydrocortisone treatment. **B:** Blot hybridized to the cDNA of pc-33. Lanes: **1**, control; **2**, after cAMP; **3**, after insulin; **4**, after hydrocortisone. In lane **5** are ^{14}C-labeled 28, 23, 18, and 16 S ribosomal RNA markers from 3T3 cells and *E. coli* (4.9, 2.9, 1.9, and 1.5 kb, respectively).

cAMP or insulin. This confirmation is shown in the Northern blots presented in Figure 1, which also illustrates the size of the cytoplasmic mRNA product of each gene relative to ribosomal RNA markers. In each case the hormonally effected increases in these mRNAs have been shown to be, primarily at least, the consequence of accelerated gene transcription [12–14], as has the developmental activation of their expression [15].

Response to Glucocorticoids in Fetal Liver

Figure 2 presents the results of hybridization assays of the amounts of TAT mRNA in livers of untreated fetuses and those injected in utero with hydrocortisone. These data confirm the low expression of this mRNA, in these analyses barely detectable, in livers of late-term fetuses. Treatment with hydrocortisone caused slight increases, most notable 3 hr after injection, but the levels attained are still very limited. This confirms that the nearly silent aminotransferase gene in fetal livers is essentially refractory to induction by glucocorticoids.

Steady-state levels of gene 33 mRNA are somewhat higher than those of TAT in fetal livers through 20 days of gestation; then they increase sharply on day 21 and again at birth, as does the rate of its transcription [15]. Expression of this gene was found to be strongly responsive to hydrocortisone treatment of fetuses at 20 and 21 days of gestation (Fig. 3), in sharp contrast to the TAT gene.

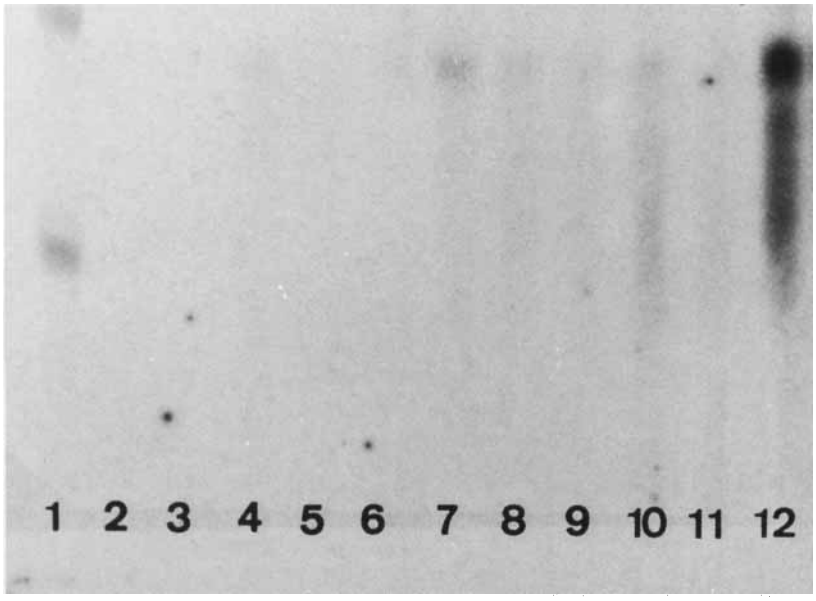

Fig. 2. Effect of hydrocortisone on TAT mRNA in fetal rat liver. Cytoplasmic poly(A)-RNAs (fetal liver, 5 μg; adult liver, 1 μg) were subjected to electrophoresis, blotted, and hybridized to the cDNA insert of pcTAT-3. RNA sources in lanes: **1**, ^{14}C-labeled *E. coli* ribosomal RNA markers; **2**, 20-day fetal liver control (injected with 0.15 M NaCl); **3** and **4**, 20-day fetuses treated with hydrocortisone for 1 and 3 hr, respectively; **5**, 21-day fetal control; **6**, **7**, **8**, **9**, and **10**, 21-day fetuses treated with hydrocortisone for 1, 3, 4.5, 6, and 12 hr, respectively; **11**, 22-day fetal control; **12**, adult control (untreated).

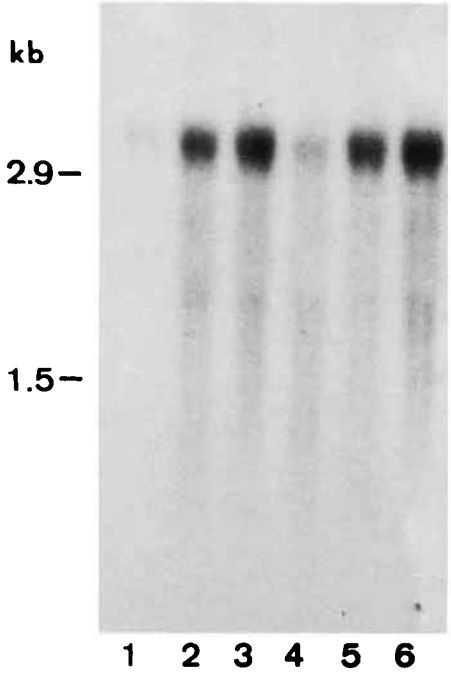

Fig. 3. Effect of hydrocortisone on gene 33 mRNA in fetal liver. Experimental details as in Figure 2 except that the blot was hybridized to ^{32}P-labeled cDNA-33. RNA sources in lanes: **1**, 20-day fetal control; **2** and **3**, 20-day fetuses treated with hydrocortisone for 1 and 3 hr, respectively; **4**, 21-day fetal control; **5** and **6**, 21-day fetuses treated with hydrocortisone for 1 and 3 hr, respectively.

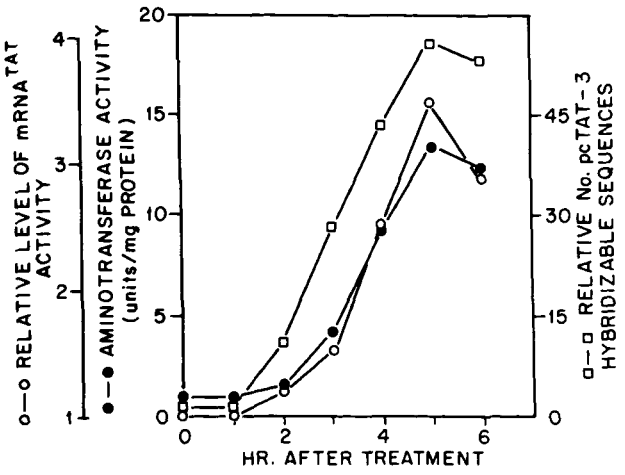

Fig. 4. Time course of induction of TAT expression by cAMP in fetal rat liver. Fetuses at 21 days were treated with 8-Br-cAMP at zero time. *Squares*, amount of TAT mRNA determined by dot hybridizations to TAT cDNA; *open circles*, TAT mRNA activity measured by translation in reticulocyte lysates as described [8]; *solid circles*, TAT enzyme activity determined by the method of Diamondstone [16].

Response to cAMP in fetal liver. In 1967 Greengard and Dewey reported that TAT activity is significantly elevated in rat fetuses treated directly with glucagon or its intracellular mediator, cAMP [5]. This has been confirmed and extended to show that aminotransferase mRNA activity is similarly increased by cAMP [6]. The time course of this response is depicted in Figure 4. Here we have repeated the earlier types of expression assays and added measurements of TAT mRNA concentration made by dot hybridizations. All parameters increase beginning 2 hr after injection and reach a max-

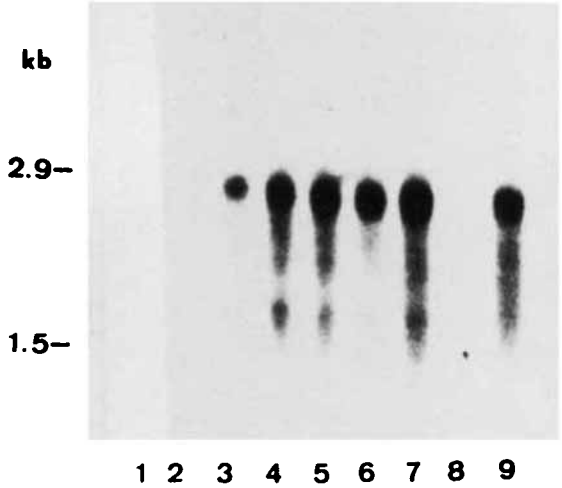

Fig. 5. Northern blot analysis of the effect of cAMP on TAT mRNA in fetal liver. Details as in Figure 2. RNA sources in lanes: **1**, 21-day fetal liver control; **2**, **3**, **4**, **5**, **6**, and **7**, 21-day fetal liver 1, 2, 3, 4, 5, and 6 hr after treatment with cAMP, respectively; **8**, 22-day fetal liver control; **9**, adult liver control (untreated).

imum at 5–6 hr, and the extent of change is comparable for each parameter. Northern blot analysis of these RNAs showed similar changes in concentrations of TAT mRNA (Fig. 5).

As shown in the Northern blot of Figure 6, expression of gene 33 is similarly responsive to cAMP in fetal liver. The time course of this response differs somewhat from that of the aminotransferase, a maximal increase in the mRNA being apparent just 2 hr after injection. These data also illustrate the earlier developmental activation of expression of this gene when compared to tyrosine aminotransferase, which remains very low until birth [4,6]. The RNA in lane 7 was from livers of untreated fetuses of 22 days gestation but not yet born; the concentration of gene 33 mRNA at this time of development is much higher than that at 21 days of fetal life (lane 1), and the latter is significantly greater than in fetuses of 20 days gestational age or earlier (cf. Fig. 3).

Response to insulin in fetal liver. In earlier work we were unable to detect any significant increase in TAT enzyme levels or of the activity of its mRNA in livers of fetal or newborn rats treated with insulin [6]. The Northern blot hybridization analysis of Figure 7 shows that this hormone, like hydrocortisone, is essentially without effect on TAT mRNA concentrations in fetal livers. Like the steroid, insulin does effect a strong enhancement in the expression of gene 33 mRNA in fetal liver (Fig. 8).

Postnatal regulation of tyrosine aminotransferase by insulin. The data of Figures 2 and 7 establish that transcription of the TAT gene is not stimulated by either hydrocortisone or insulin in fetal liver. It was shown earlier that the capacity of this gene to respond to glucocorticoids is attained concomitantly with its developmental

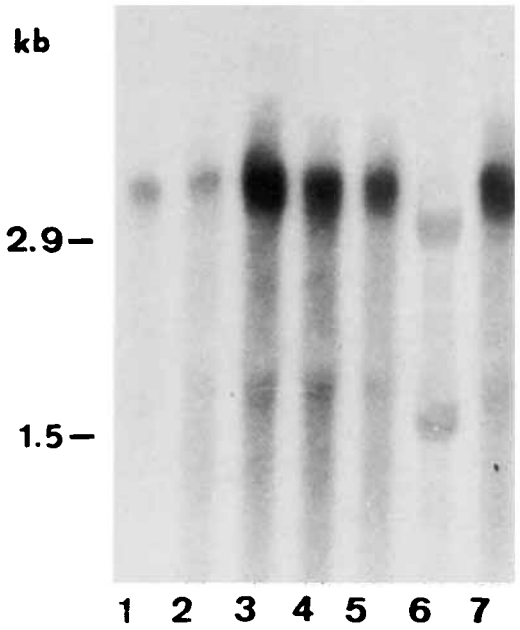

Fig. 6. Effect of cAMP on gene 33 mRNA in fetal liver. Details as in Fig. 3. RNA sources in lanes: **1**, 21-day fetal control; **2**, **3**, **4**, and **5**, 21-day fetuses treated with 8-Br-cAMP for 1, 2, 3, and 4 hr, respectively; **6**, ^{14}C-labeled 2.9- and 1.5-kb ribosomal RNA markers; **7**, 22-day fetal control.

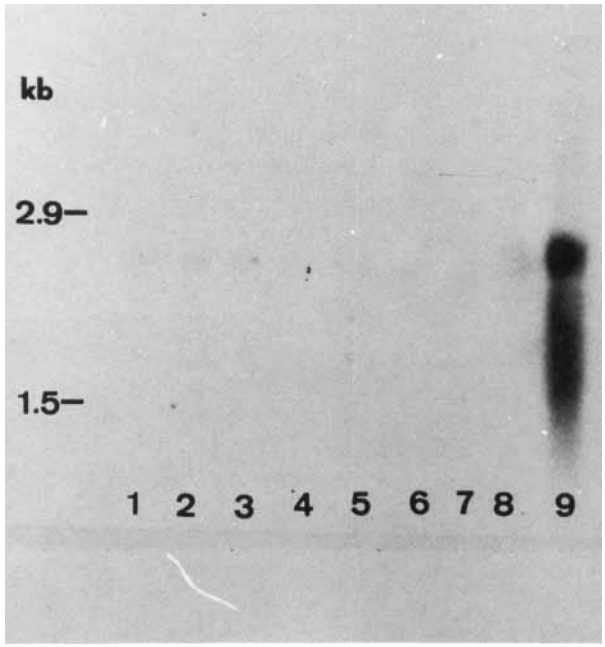

Fig. 7. Effect of insulin on TAT mRNA in fetal liver. Details as in Figure 2. RNA sources in lanes: **1**, 20-day fetal control; **2** and **3**, 20-day fetuses treated with insulin for 1 and 3 hr, respectively; **4**, 21-day fetal control; **5**, **6**, **7**, and **8**, 21-day fetuses treated with insulin for 1, 2, 3, and 4 hr, respectively; **9**, adult control (untreated).

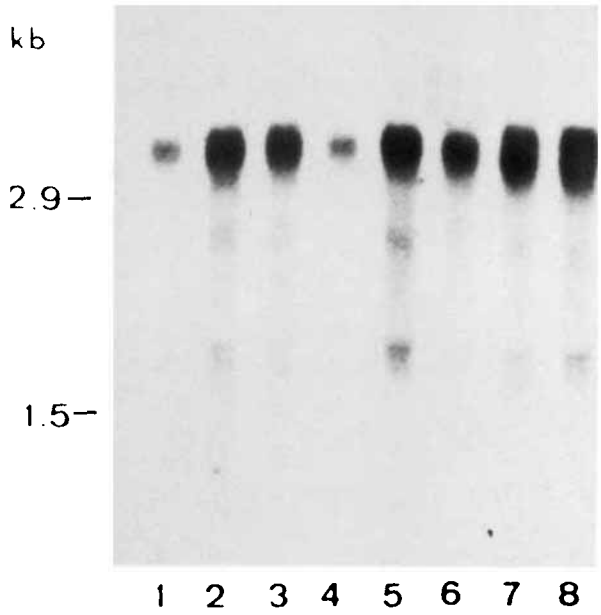

Fig. 8. Effect of insulin on gene 33 mRNA in fetal liver. Details as in Figure 3. RNA sources in lanes: **1**, 20-day fetal control; **2** and **3**, 20-day fetuses treated with insulin for 1 and 3 hr, respectively; **4**, 21-day fetal control; **5**, **6**, **7**, and **8**, 21-day fetuses treated with insulin for 1, 2, 3, and 4 hr, respectively. The fetal liver RNA preparations used here are the same as those analyzed for TAT expression in Figure 7.

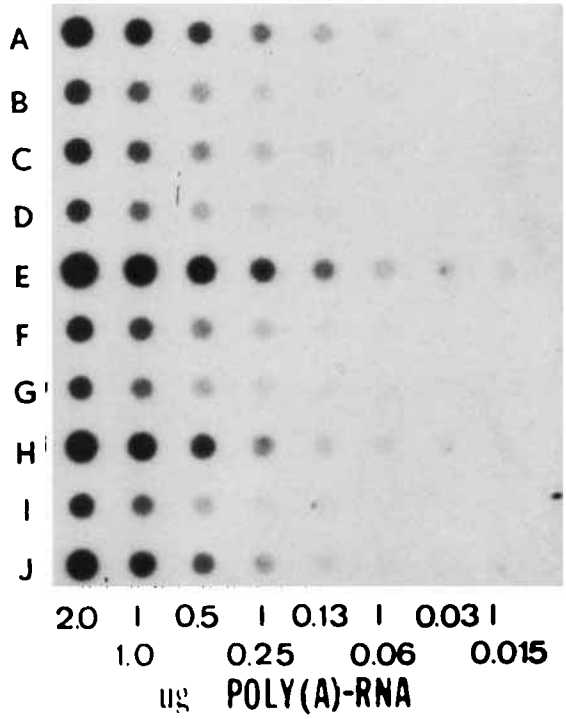

Fig. 9. Dot hybridization analysis of the effect of insulin on TAT mRNA in livers of neonatal rats. Poly(A)-RNAs in the amounts indicated were hybridized to the cDNA of pcTAT-3; the RNAs were prepared from pooled livers of at least six neonates in each group. **A**, 20 hr postnatal, 0.15 M NaCl-injected control; **B**, 20 hr postnatal, treated for 1 hr with 1 unit/100 g insulin; **C** and **D**, 24 hr postnatal, control and insulin-treated, respectively; **E** and **F**, 48 hr postnatal, control and insulin-treated, respectively; **G** and **H**, 72 hr postnatal, control and insulin-treated, respectively; **I** and **J**, 96 hr postnatal, control and insulin-treated, respectively.

activation at birth; indeed, the large burst in TAT expression occurring in the first hours after birth can be attributed in part to induction by endogenous glucocorticoids [6]. To determine the point in development at which this expression becomes responsive to insulin we examined RNAs from livers of neonatal rats by dot hybridization (Fig. 9). Levels of TAT enzyme and mRNA are quite variable during the first days after birth, and we analyzed pooled livers from several individuals of comparable age to minimize this variation. We were surprised to find that insulin reduced the concentration of TAT mRNA in neonates 20–48 hr after birth, an effect especially notable in the 48-hr sample (lanes E, F) and as yet completely inexplicable, but confirmed by comparable decreases in levels of the enzyme (not shown). Insulin treatment effected an increase in the mRNA in the samples taken 72 and 92 hr postnatally, indicating that the induction response capacity to this hormone (again confirmed by enzyme assays) is not acquired until several days after developmental activation of aminotransferase expression.

DISCUSSION

Expression of gene 33 in fetal liver is stimulated by each of the hormonal regulators that accelerate its transcription in adults, establishing that the fetal liver contains the

fundamental elements required for an induction response to glucocorticoids, insulin, or cAMP. Yet tyrosine aminotransferase, regulated in virtually identical fashion by these agents in adults, is responsive only to cAMP in the fetus, remaining refractory to glucocorticoid control until birth and to induction by insulin until several days postpartum. Thus there is a degree of specificity in the development of the capacity of individual genes to respond to hormonal regulators. A similar conclusion was reached in a study of estrogen-responsive genes in embryonic chick liver by Elbrecht et al. [17], who found that two such genes, coordinately regulated by estrogen in adult liver, acquired responsiveness to this hormone at different times during development.

Developmental changes in expression of TAT have been studied in some depth. In livers of late-term fetuses expression is very low but detectable and in this quiescent state is refractory to control by glucocorticoids (or insulin) but responsive to induction by cAMP. It is significant that this response to cAMP, and that of gene 33 to each of the hormonal inducers in fetal liver, is quantitatively comparable to those observed after treatment of adults with these hormonal agents when considered in terms of the extent of increase over the level of expression prior to treatment. But the absolute levels of expression attained after hormone treatment in fetal liver are still well below those observed in adults, indicating that the quiescent state of expression remains limiting, perhaps indicative of limited promoter activity of these genes in fetal liver. These results are in accord with the realization that hormonal control of gene expression involves enhancer elements [18]; the effectiveness of such elements would be expected to be limited by primary control at the promoter level.

At birth there is an abrupt increase in aminotransferase expression and the capacity to respond to glucocorticoids occurs concomitantly. The mechanism(s) involved in this developmental transition is not understood, but significant insight is being gained through analyses of mutant mice carrying deletions which include the *c* (albino) locus on chromosome 7. Newborn mice homozygous for deletions such as those designated c^{14CoS} or c^{3H} survive only briefly and exhibit impaired differentiation of the liver, apparently owing in part to the lack of one or more *trans*-acting regulatory genes normally present in the deleted region [summarized in 19,20]. The impairment includes the failure of genes such as those encoding tyrosine aminotransferase or phosphoenolpyruvate carboxykinase to become activated at birth. Recent work utilizing specific hybridization assays has confirmed that the quiescent state of expression of these genes is maintained in newborns homozygous for these deletions and that they remain refractory to glucocorticoid control [21,22]. Expression of gene 33, shown here to be responsive to hydrocortisone in fetal liver, is not affected by the *c* locus region deletion in mice [15]. Thus a reasonable case can be made for the suggestion that glucorticoid control of some genes (e.g., the aminotransferase and carboxykinase genes) but not others (e.g., gene 33) requires the product of a *trans*-acting regulatory gene as well as interaction of the steroid-receptor complex with glucocorticoid regulatory elements in DNA. A recent study by Cordingley et al. can be interpreted as providing support for this suggestion. These authors found that interaction of the steroid-receptor complex with the glucocorticoid regulatory element of the mouse mammary tumor virus activates transcription by recruitment of transcription factors at the gene promoter [23]. If the product of the putative *trans*-acting regulatory gene near the *c* locus is a gene-specific transcription factor synthesized at birth, as seems quite possible, the refractory state of the aminotransferase in fetal liver and acquisition of glucocorticoid responsiveness at birth could be understood as a requirement for an interaction of the sort described by Cordingley et al.

Activation at birth of aminotransferase transcription does not confer responsiveness to induction by insulin; this capacity is not attained until several days postpartum. The mechanisms by which insulin binding to its membrane receptor are transduced into control of expression of specific genes or into any of the multiple intracellular effects of insulin have not been defined. A number of signal-mediating mechanisms have been suggested, including internalization and translocation to the nucleus of the hormone-receptor complex or fragments of it [24,25], a cascade of protein phosphorylations following activation of the receptor's protein kinase activity [26], and the generation of both glycopeptide [27] and glycolipid [28,29] mediators. It is possible that gene 33 and tyrosine aminotransferase inductions by insulin require different mediators, that involved in controlling the latter gene not being generated in response to the hormone until some postnatal developmental change has occurred. An alternative and perhaps more likely explanation is that presumptive insulin regulatory elements in or near the aminotransferase gene must undergo a developmentally controlled modification (e.g., demethylation) in order to become responsive to an existing mediator; such a modification could be involved in the attainment of glucocorticoid responsiveness as well.

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