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# Role of the Isoforms of CCAAT/Enhancer-binding Protein in the Initiation of Phosphoenolpyruvate Carboxykinase (GTP) Gene Transcription at Birth*

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**The gene for phosphoenolpyruvate carboxykinase (PEPCK), a target of CCAAT/enhancer-binding protein-α (C/EBPα) and -β (C/EBPβ), begins to be expressed in the liver at birth. Mice homozygous for a deletion in the gene for CEBPα (C/EBPα$^{-}$/_ mice) die shortly after birth of hypoglycemia, with no detectable hepatic PEPCK mRNA and negligible hepatic glycogen stores. Half of the mice homozygous for a deletion in the gene for CEBPβ (C/EBPβ$^{-}$/_ mice) have normal glucose homeostasis (phenotype A), and the other half die at birth of hypoglycemia due to a failure to express the gene for PEPCK and to mobilize hepatic glycogen (phenotype B). Insulin deficiency induces C/EBPα and PEPCK gene transcription in the livers of 19-day fetal rats, whereas dibutyryl cyclic AMP ($Bt_2$cAMP) increases the expression of the gene for C/EBPβ and causes a transient burst of PEPCK mRNA. $Bt_2$cAMP induces PEPCK mRNA in the livers of fetal C/EBPα$^{-}$/_ mice, but at only 20% of the level of control animals; however, there is no induction of PEPCK mRNA if the cyclic nucleotide is injected into C/EBPα$^{-}$/_ mice immediately after delivery. The expression of the gene for C/EBPβ is markedly induced in the livers of C/EBPα$^{-}$/_ mice within 2 h after the administration of $Bt_2$cAMP. C/EBPβ$^{-}$/_ mice injected at 20 days of fetal life with $Bt_2$cAMP have a normal pattern of induction of hepatic PEPCK mRNA. In C/EBPβ$^{-}$/_ mice with phenotype B, the administration of $Bt_2$cAMP immediately after delivery induces PEPCK mRNA, causes the mobilization of hepatic glycogen, and maintains normal glucose homeostasis for up to 4 h (duration of the experiment). We conclude that C/EBPα is required for the cAMP induction of PEPCK gene expression in the liver and that C/EBPβ can compensate for the loss of C/EBPα if its concentration is induced to appropriate levels.**

The stepwise differentiation of gene expression has been intensively studied (1, 2). The liver-specific expression of genes is defined by a combination of hepatic enriched nuclear transcriptional activators that bind their cognate DNA motifs in the target genes. Four families of such activators (HNF-1,[1] -3, and -4 and the C/EBP family of transcription factors), whose members bind to the same DNA motif, have been characterized to date. Regulatory regions of liver-specific genes include a combination of recognition motifs of several distinct activators from one or more of these families. The coexistence of a specific combination of these activators in the cell and their binding to the cognate target motifs are essential for the liver-specific gene transcription (3–5). Thus, the ordered initiation of transcription of each liver-specific gene depends on the developmental appearance of the appropriate set of transcriptional activators that appear sequentially during liver development in correlation with the initiation of expression of their target genes (6). For example, members of the HNF-3 family appear in primordial hepatocytes (7), whereas two important members of the C/EBP family (C/EBPα and C/EBPβ) are transcribed initially in the liver during the last trimester of development (8, 9), and D Binding Protein (DBP) is present only after birth (10).

The signals that induce the appearance of these transcription factors during liver development have not been identified. The involvement of hormone-mediated signal transduction has been previously suggested in the case of C/EBPα (11) and demonstrated for adipocyte differentiation in cultured cells, where the expression of the genes for members of the C/EBP family is induced (8, 12, 13). There is also a differential modulation of members of this family during the growth and arrest phases of liver regeneration (14, 15). The accumulation of at least two members of the C/EBP family in the liver (8, 9, 16), during a period of profound changes in the hormonal environment of the neonate, suggests that hormones might be involved in triggering the expression of the genes for these two transcription factors. This has been reinforced by our previous studies that have implicated members of the C/EBP family in the onset of transcription of the gene for the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (PEPCK; EC 4.1.1.32) at birth (17). Although this gene is not expressed in the fetal liver and appears at birth (18), it can be prematurely activated by increasing the glucagon/insulin ratio to a state that is similar to that which prevails at birth (19). It is possible to cause the premature activation of PEPCK gene expression by injecting fetuses *in utero* with $Bt_2$cAMP or by administering streptozotocin, which causes insulin deficiency (20, 21). Fur-

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[1] The abbreviations used are: HNF, hepatic nuclear factor; C/EBP, CCAAT/enhancer-binding protein; PEPCK, phosphoenolpyruvate carboxykinase; $Bt_2$cAMP, dibutyryl cyclic AMP; kb, kilobase pair; CRE, cAMP response element; DBP, D binding protein.

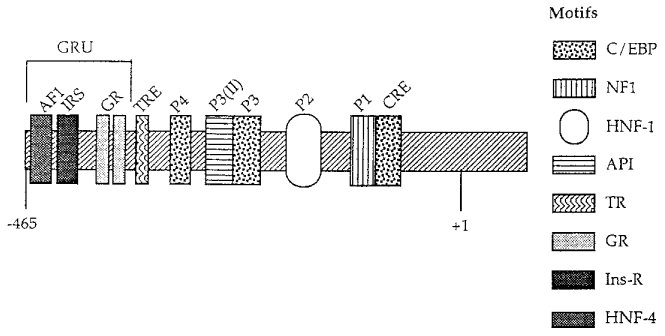


FIG. 1. **Schematic illustration of the regulatory sites in the promoter region of the PEPCK gene.** The *horizontal box* indicates the PEPCK promoter region from the transcription start site at position +1 to position −465. The *vertical boxes* indicate binding sites termed P1–P4 and the following specific sites: the CRE, the thyroid hormone response element (*TRE*) (59), the glucocorticoid receptor-binding site (*GR*) (60), the insulin regulatory sequence (*IRS*) (61), and AF1 (homologous to the AF1 element of the apoC-III gene). The glucocorticoid regulatory unit (*GRU*) is composed of the two glucocorticoid receptors, the insulin regulatory sequence, and AF1 (60). The factors binding to each motif are indicated on the right. The CRE binds the cAMP response element-binding protein and AP1 in addition to C/EBP (23, 51). *NF1*, nuclear factor-1; *TR*, thyroid hormone receptor; *GR*, glucocorticoid receptor; *Ins-R*, insulin receptor.

thermore, the chromatin conformation of the PEPCK gene in the liver undergoes a gradual transition from a "compact" conformation on day 19 of gestation to an "open" conformation just prior to the initiation of its transcription at birth (18). Injecting 19-day fetal rats with $Bt_2$cAMP induced both this process and the expression of the gene for PEPCK in the fetal liver (18). There may thus be a gradual assembly of nuclear factors onto the PEPCK promoter during development, which is prompted by alterations in the concentration of hepatic cAMP. DNase I footprinting analysis of the PEPCK promoter using nuclear extracts from the livers of fetal, newborn, and adult rats indicated distinct binding interactions that were developmentally regulated, *i.e.* they were fully present in nuclear proteins extracted from the livers of newborn and adult animals, but deficient in liver extracts from fetal rats (17). Nuclear proteins from the livers of newborn and adult rats protected all of the eight binding sites in the PEPCK promoter that have been previously mapped by Roesler *et al.* (22). These included binding to the recognition sites for nuclear factor-1 and cAMP response element-binding protein and to the sites for the hepatocyte-enriched factors HNF-1 and HNF-4 and for three C/EBP motifs (Fig. 1). However, nuclear proteins from the fetal liver interacted poorly with those sites in the PEPCK promoter that bind C/EBP (17). This suggested that whichever member of the C/EBP family binds to the PEPCK promoter, it is developmentally regulated, *i.e.* it is deficient in rat liver at 18 days of fetal life and accumulates at birth. The pattern of development of the binding activity exactly coincides with the appearance of C/EBP$\alpha$ in the liver (8).

The importance of C/EBP$\alpha$ in the control of glucose homeostasis was clearly demonstrated in the studies of Wang *et al.* (24) using mice homozygous for a deletion in the gene for this transcription factor. These mice die at birth of profound hypoglycemia; they have no mRNA for PEPCK or glucose-6-phosphatase in their livers and have only marginal levels of hepatic glycogen present at birth. Mice with a deletion in the gene for C/EBP$\beta$ have a more complex pattern of regulation of glucose homeostasis. Only half of the expected mendelian ratio of C/EBP$\beta^{-}$/_ mice from the mating of mice heterozygous for the deletion in the C/EBP$\beta$ gene are alive at weaning; these animals are more susceptible to infections (25, 26). Here we report that the other 50% of C/EBP$\beta$-deficient mice die of profound hypoglycemia and that they are unable to induce the initial transcription of the gene for PEPCK in the liver at birth and hence are unable to synthesize hepatic glucose during the critical perinatal period. The availability of these "knockout" animal models provided us with a unique tool to study the effect of C/EBP$\alpha$ and C/EBP$\beta$ on the development of PEPCK gene transcription in particular and on the normal pattern of development of glucose homeostasis in general. Our results demonstrate that C/EBP$\alpha$ is required for the normal induction of hepatic PEPCK gene transcription by cAMP during the perinatal period; C/EBP$\beta$ is not required for cAMP induction of PEPCK gene transcription before birth, but is necessary for the normal development of PEPCK gene expression in the liver immediately after birth. There is thus a complex interaction between C/EBP$\alpha$ and C/EBP$\beta$ that results in the regulation of PEPCK gene transcription during the perinatal period.

## EXPERIMENTAL PROCEDURES

*Materials*—$Bt_2$cAMP, theophylline, streptozotocin, guanidine thiocyanate, and cesium chloride were purchased from Sigma. [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) and [$^{32}$P]UTP (400 Ci/mmol) was from NEN Life Science Products. An ECL Western blot analysis kit was purchased from Amersham Corp.

*Animals*—Sabra rats of Wistar origin were provided by the Harland Breeding Farm at the Hebrew University Medical School. Adult rats were 6-week-old males. Rats pregnant for 18 and 19 days were anesthetized with ether; uteruses were exposed by partial laparectomy; and fetuses were injected through the uterine wall with 25 $\mu$g of $Bt_2$cAMP and 25 $\mu$g of theophylline in 15 ml of saline. Fetal rats at 18 days of gestation were injected with $Bt_2$cAMP and theophylline as noted above and killed 24 h later. Animals at 19 days of gestation were killed either 40 min (0.7 h) or 3 h after injection. Immediately upon termination of the injections, the fetuses were put back into the dam's abdomen, and the wall was sutured. Control animals were surgically treated without any injections. Insulin deficiency was generated by streptozotocin treatment as described previously (18). Briefly, streptozotocin (480 mg/ml of 10 mM sodium citrate (pH 4.5) in 0.9% NaCl) was freshly dissolved immediately before the injection. Fetal rats were injected *in utero* with 5 ml of streptozotocin once daily on 2 successive days.

Mice homozygous for deletions in the genes for C/EBP$\alpha$ and C/EBP$\beta$ were generated as described by Wang *et al.* (24) and by Screpanti *et al.* (25), respectively. For our studies, we used mice heterozygous for the deletion in the respective gene to generate animals. At 20 days of fetal life, the mice were injected with 125 mg of $Bt_2$cAMP/kg of body weight (27) *in utero* as described above for rats and killed 2 h later. The livers were removed, and RNA was isolated. Alternatively, the mice were delivered by cesarean section at 20 days of fetal life and then injected with 125 mg of $Bt_2$cAMP/kg of body weight; the animals were maintained in a humidicrib at 37 °C for the periods indicated in the figure legends before being used for the analysis of blood glucose using a Beckman commercial glucose analyzer and for the determination of the concentration of specific mRNA in the liver.

*Molecular Probes*—The PEPCK cDNA probe was a 1.6-kb *Pst*I fragment (28). The C/EBP$\alpha$ cDNA probe was a 2.1-kb *Bam*HI-*Hin*dIII fragment (29), and the C/EBP$\alpha$ genomic fragment used to screen for C/EBP$\alpha^{-}$/_ mice was a 1.8-kb *Eco*RI-*Hin*dIII fragment of the C/EBP$\alpha$ gene (24). The C/EBP$\beta$ cDNA probe was a 0.67-kb *Pst*I mouse fragment (30) or a 0.7-kb *Bam*HI mouse genomic fragment (25), which was used to screen for C/EBP$\beta^{-}$/_ mice. The superoxide dismutase cDNA was a 0.62-kb *Pst*I human fragment (31), and the albumin cDNA was a 1.2-kb *Hin*dIII fragment from the rat (32). The actin probe used in this study was a 2.1-kb *Bam*HI fragment of human actin cDNA (33).

*RNA Analysis*—Northern blot analysis was performed as described previously (34) using either 5 $\mu$g of poly(A)$^+$ RNA or 20 $\mu$g of total RNA. Briefly, total RNA from tissues was extracted with guanidine thiocyanate and centrifuged through a CsCl cushion following the method of Chirgwin *et al.* (35). Poly(A)$^+$ RNA was isolated by affinity chromatography on oligo(dT)-cellulose (36). For the analysis of PEPCK mRNA in the livers of the mice used in this study, total RNA was used directly for Northern analysis.

*Run-on Transcription Assay*—Nuclei were prepared from the livers of adult and 19-day fetal rats as described by Meisner *et al.* (37). Elongation of nascent RNA chains from the isolated nuclei was carried out essentially as described by Schibler *et al.* (38), except that heparin sulfate was not used and there was a lower concentration of $(NH_4)_2SO_4$

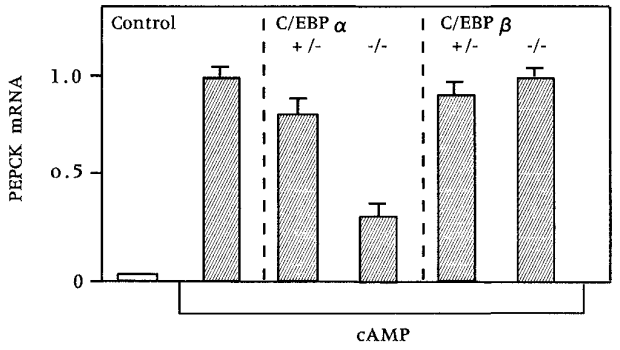


FIG. 2. **Effect of $Bt_2$cAMP on the expression of the gene for PEPCK in the livers of 20-day fetal mice homozygous and heterozygous for deletions in the gene for either C/EBPα or C/EBPβ.** Mice at 20 days of fetal life that were homozygous (C/EBP$\alpha^-$/_) or heterozygous (C/EBP$\alpha^+$/_) for a deletion in the gene for C/EBPα and mice homozygous (C/EBP$\beta^-$/_) or heterozygous (C/EBP$\beta^+$/_) for a deletion in the gene for C/EBPβ were injected *in utero* with saline or $Bt_2$cAMP (125 mg/kg of body weight) as described in detail under "Experimental Procedures." Controls were normal mice also at 20 days of fetal life. The mice were killed 2 h later, the livers were removed, and RNA was isolated. The level of PEPCK mRNA was determined by Northern blotting and was standardized relative to the level of actin mRNA. The *bars* represent the mean ± S.E. for four to six mice in each group.

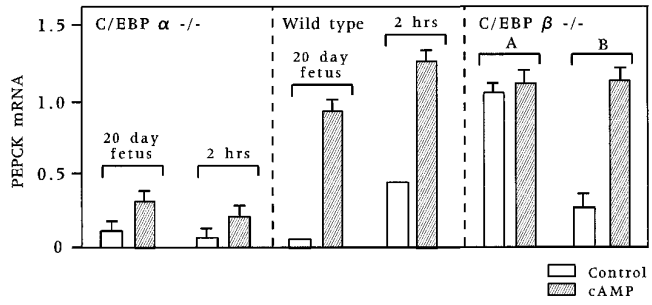


FIG. 3. **Effect of $Bt_2$cAMP on the expression of the gene for PEPCK in the livers of mice homozygous for deletions in the genes for C/EBPα and C/EBPβ and in normal mice 2 h after delivery.** Mice at 20 days of fetal life that were homozygous for a deletion in the genes for C/EBPα and C/EBPβ were delivered by cesarean section and either injected interparietally with $Bt_2$cAMP (125 mg/kg of body weight; *cAMP*) or not injected (*Control*) as described in detail under "Experimental Procedures." Two hours later, the animals were killed, the livers were removed, and RNA was isolated. The level of PEPCK mRNA was determined by Northern blotting and was standardized relative to the level of actin mRNA. For comparison, wild-type mice were included to demonstrate the effect of $Bt_2$cAMP on the level of hepatic PEPCK mRNA in the liver. The effect of $Bt_2$cAMP on the induction of PEPCK gene expression in the livers of normal (wild-type) mice is included for comparison. The *bars* represent the mean ± S.E. for three to five mice in each group.

(35 μM) in the reaction mixture. Labeled RNA was extracted from the reaction mixture (39) and hybridized to DNA, which was bound to GeneScreen Plus (NEN Life Science Products). Immobilization of linearized plasmid DNA and hybridization were performed according to Aloni *et al.* (40). The hybridization signal was quantitated using a PhosphorImager.

*Western Blot Analysis*—Nuclear extracts from the livers of 19-day fetal and adult rats were prepared by the method of Gorski *et al.* (41), with minor modifications (17). Western blot analysis was performed according to Towbin *et al.* (42). Briefly, 20-mg nuclear extracts were separated on 12% SDS-polyacrylamide gels, followed by transfer to filters that were first immunoreacted with a polyclonal antibody to C/EBPα (kindly provided by Dr. Steven L. McKnight, University of Texas, Southwest Medical Center) and then stripped of the radioactive probe after detecting the signal and reacted with a polyclonal antibody to C/EBPβ (kindly provided by Dr. D. Ron, New York Medical Center). The commercial ECL Western blot analysis kit was used for detection of the signals.

## RESULTS

To determine the role of C/EBPα and C/EBPβ in the expression of the gene for PEPCK in the liver during development, we have used mice that contain deletions in the genes for these transcription factors. The level of PEPCK mRNA in the livers of 20-day fetal mice with a deletion in the genes for C/EBPα and C/EBPβ was determined 2 h after the administration of $Bt_2$cAMP to animals *in utero* (Fig. 2). PEPCK mRNA in the livers of control mice at day 20 of fetal life was not detectable, but injecting the mice with $Bt_2$cAMP resulted in a marked induction of transcription of the PEPCK gene. With C/EBP$\alpha^-$/_ mice, the induction of expression of the PEPCK gene in the liver by $Bt_2$cAMP was ~20% of that noted with wild-type mice or with animals heterozygous for a deletion in the gene for C/EBPα. In contrast, $Bt_2$cAMP caused the expected induction of PEPCK mRNA in the livers of mice either heterozygous or homozygous for a deletion in the gene for C/EBPβ. Thus, C/EBPα is required for the normal full induction of PEPCK by cAMP in the livers of fetal mice.

We next determined the relative importance of the two C/EBP isozymes in the expression of the gene for PEPCK in the livers of mice in the immediate postnatal period (Fig. 3). C/EBP$\alpha^-$/_ mice had barely detectable levels of PEPCK mRNA in their livers 2 h after birth. The administration of $Bt_2$cAMP caused a marginal induction of PEPCK gene expression in the livers of these mice 2 h after the administration of the cyclic nucleotide. For comparison, the effect of $Bt_2$cAMP on the induction of PEPCK mRNA in the livers of 20-day fetal mice with a deletion in the gene for C/EBPα is included. Fig. 3 (*middle panel*) shows the expected induction by $Bt_2$cAMP of PEPCK gene expression in the livers of wild-type mice either at 20 days of fetal development or at 2 h after birth. In this case, there were only marginal levels of PEPCK mRNA in the livers of the mice, and the administration of $Bt_2$cAMP resulted in a rapid and marked accumulation of PEPCK mRNA in the liver within 2 h. The situation with the C/EBP$\beta^-$/_ mice is more complex. These animals have two distinct phenotypes; mice with phenotype A have normal glucose homeostasis, but are more susceptible to infections (25, 26). Mice with phenotype B have severe hypoglycemia: 10–16 mg/dl glucose as compared with 60 mg/dl in the blood of normal mice at the same age (data not shown) and no detectable PEPCK mRNA. These mice also have a hepatic glycogen concentration of ~3%, which is in the same general range as noted for normal littermates of the same age (data not shown). When C/EBP$\beta^-$/_ mice with phenotype B were injected with $Bt_2$cAMP 2 h after delivery, there was a marked increase in the expression of the gene for PEPCK within 2 h of the injection of the cyclic nucleotide, with a resultant accumulation of PEPCK mRNA in the liver (Fig. 3).

We next determined the time course of $Bt_2$cAMP induction of expression of the genes for C/EBPα, C/EBPβ, and PEPCK in the livers of 19-day fetal rats. RNAs for both isoforms[2] of C/EBP were detectable in the fetal liver, albeit at low levels relative to those in the livers of adult rats. The injection of $Bt_2$cAMP into fetuses had no effect on C/EBPα mRNA, but induced the level of C/EBPβ mRNA. Although the concentration of C/EBPβ mRNA increased only 5-fold, it shared the same transient pattern of induction as that of PEPCK mRNA (Fig. 4). As anticipated, PEPCK mRNA was markedly induced in the livers of these fetuses by 40 min (0.7 h in Fig. 4) after the administration of $Bt_2$cAMP and increased further over the next 3 h. By 24 h, PEPCK mRNA was no longer detectable in the liver (Fig. 4). The levels of PEPCK mRNA at the peak exceeded

[2] The term isoforms, as used in this paper, refers to the various members of the C/EBP family of transcription factors (*i.e.* C/EBPα, C/EBPβ, and D binding protein).

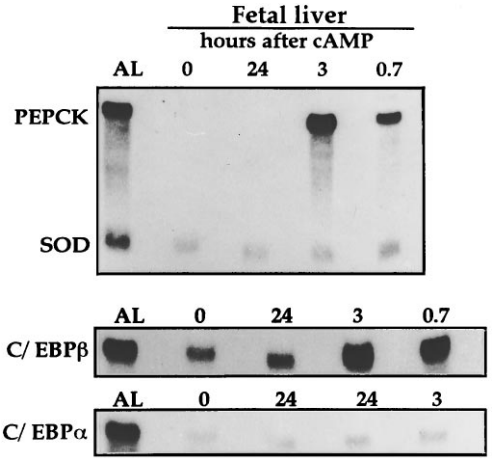


FIG. 4. **Effect of development and $Bt_2$cAMP administration on the levels of C/EBP$\alpha$, C/EBP$\beta$, and PEPCK mRNAs in the livers of fetal and adult rats.** Northern blot hybridization was carried out using 5 $\mu$g of poly(A)$^+$ RNA in each lane. RNA was isolated from adult liver (*AL*) and from the livers of 20-day fetal rats at 0, 0.7 (40 min), 3, and 24 h after $Bt_2$cAMP treatment, as indicated, and hybridized with PEPCK and superoxide dismutase (*SOD*) cDNAs. After the first hybridization, the blots were rehybridized with C/EBP$\alpha$ and C/EBP$\beta$ cDNAs.

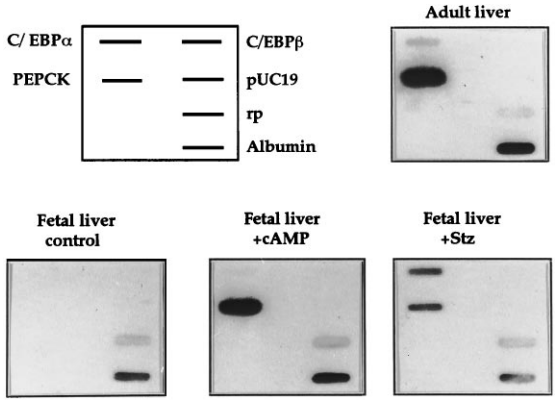


FIG. 5. **Determination of the rate of transcription of the genes for PEPCK, C/EBP$\alpha$, and C/EBP$\beta$ using nuclei isolated from the livers of fetal and adult rats.** Run-on transcription assays were carried out using nuclei from adult liver or from the livers of 19-day fetal rats before (*control*) and 40 min after *in utero* injection of $Bt_2$cAMP (+*cAMP*) or insulin deficiency caused by streptozotocin injection for 2 successive days (+*Stz*). The newly synthesized [$^{32}$P]RNA was hybridized with filters containing a 2.5 $\mu$g/lane concentration of plasmid DNA carrying the specific cDNAs as indicated by the index in the *upper left box*, including the pUC19 plasmid used to indicate the background signal. *rp* designates pooled cDNAs (a total of 10 $\mu$g/lane) of five different rat ribosomal proteins. These experiments were repeated three times with quantitatively similar findings.

those noted in the livers of control fetuses by a factor of at least 500, as judged by comparison of the autoradiographic signals at different exposure times.

Run-on transcription assays were then performed to assess whether the premature induction of PEPCK gene expression in the livers of 19-day fetal rats resulted from the initiation of gene transcription (Fig. 5). Pooled cDNAs of five different ribosomal proteins (S4, L5, L7, L18A, and L19), whose transcription rates in the liver are stable later in development (40), were used to normalize the transcriptional activities of the various nuclear preparations. In addition, since the rate of albumin gene expression on a per hepatocyte basis is the same in the fetal and adult livers (43), its rate of transcription was used to determine the fraction of the parenchyma-derived nuclei in the fetal liver compared with that in the adult liver. The rate of transcription of the albumin gene in the fetal liver was 30–40% of that in the adult liver. On this basis, we estimated that the parenchymal cells in the livers of 19-day fetal rats constitute ~30–40% of the liver cells. The injection of $Bt_2$cAMP into these fetuses resulted in the onset of transcription of the PEPCK gene in the liver (within 40 min after the injection), which exceeded by at least 200-fold that of control animals of the same age; the level of transcription was 25% of the rate obtained with nuclei from the livers of adult rats. The marginal transcription rate of the C/EBP$\beta$ gene in nuclei from either the adult or fetal liver precluded any conclusion regarding an effect of cAMP on the transcription of this gene (Fig. 5).

In contrast to the gene for C/EBP$\beta$, transcription of the gene for C/EBP$\alpha$ was readily detected in nuclei from the adult liver, but not in those from the fetal liver whether before or after injection of $Bt_2$cAMP (Fig. 5). At birth, there is a relative insulin deficiency; when streptozotocin was injected into fetal rats, it caused an induction of PEPCK gene expression in the fetal liver that was independent of cAMP (16, 44). Northern blot analysis of total RNA from fetuses treated with streptozotocin indicated the presence of PEPCK mRNA, the estimated level of which reached ~5% of that found in adult tissues (data not shown). The rate of transcription of the PEPCK gene after the administration of streptozotocin was ~10% of that noted after injection of $Bt_2$cAMP (Fig. 5), corroborating the lower increase in the abundance of PEPCK transcripts. More important, streptozotocin treatment greatly induced C/EBP$\alpha$ gene transcription, surpassing by ~3-fold the rate of its transcription by nuclei from the livers of adult animals (Fig. 5). Therefore, insulin deficiency clearly affected C/EBP$\alpha$ gene transcription in a manner independent of cAMP.

We next determined the effect of $Bt_2$cAMP on the level of the two C/EBP transcription factors by Western blot analysis. Nuclear extracts were prepared from the livers of 19-day fetuses at various times after injection of $Bt_2$cAMP. C/EBP$\alpha$ was abundant in nuclei from the livers of adult rats, but was barely detected in liver nuclei from either control or $Bt_2$cAMP-treated fetuses (Fig. 6). In contrast, the concentration of C/EBP$\beta$ in the fetal liver increased 3–4-fold by 40 min and 3 h after $Bt_2$cAMP treatment of the fetuses from barely detectable levels in the untreated fetuses; the level of C/EBP$\beta$ subsided by 24 h after the treatment. Thus, the transient increase in this protein correlated with the observed increase in its mRNA. At its peak, the induced level of nuclear C/EBP$\beta$ in the livers of fetal rats reached 10–20% of that noted in nuclei from the livers of adult animals (Fig. 6). This is most probably an underestimation, considering that fetal hepatocytes constitute only 30–40% of the cells found in the livers of adult animals.

It is possible that the injection of $Bt_2$cAMP induces a compensatory rise in the concentration of one of the C/EBP isoforms in the livers of the mice deficient in the gene for the other isoform. This could partially compensate for the deletion in the gene for C/EBP in the these mice. We therefore tested the effect of $Bt_2$cAMP on the expression of the gene for C/EBP$\alpha$ (or C/EBP$\beta$) in the livers of 19-day fetal mice deficient in the other isoform (Fig. 7). The level of C/EBP$\beta$ mRNA was induced nearly 10-fold in the livers of mice homozygous for a deletion in the gene for C/EBP$\alpha$ within 2 h after the injection of $Bt_2$cAMP into the mice *in utero*. However, the increase in the concentration of C/EBP$\beta$ mRNA in the livers of wild-type mice after $Bt_2$cAMP administration was only 3-fold; this difference is due to the fact that the relative concentration of C/EBP$\beta$ in the livers of C/EBP$\alpha^-$/_ mice is ~30% of that noted in the livers of wild-type animals. Since C/EBP$\beta$ also binds to the CRE of the PEPCK promoter (45), it is possible that its induction may be partially responsible for the observed increase in PEPCK mRNA in the livers of C/EBP$\alpha^-$/_ mice after the administra-

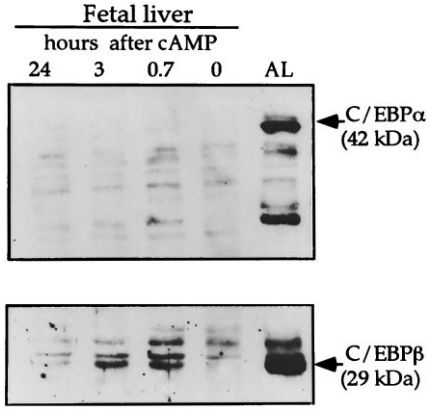


FIG. 6. **Effect of $Bt_2$cAMP on the levels of C/EBP$\alpha$ and C/EBP$\beta$ as determined by Western blot analysis.** The levels of C/EBP$\alpha$ and C/EBP$\beta$ in nuclei from adult liver (*AL*) and from the livers of 19-day fetal rats before (0 h) and 0.7 (40 min), 3, and 24 h after $Bt_2$cAMP injection as indicated were determined by Western blot analysis. Nuclear proteins (20 $\mu$g) were separated on 12% SDS-polyacrylamide gels, followed by transfer to filters that were first reacted with C/EBP$\alpha$ antibody. This antibody was then stripped off after signal detection, and the filters were reacted with an antibody to C/EBP$\beta$. A commercial ECL detection kit was used for detection of the signals.

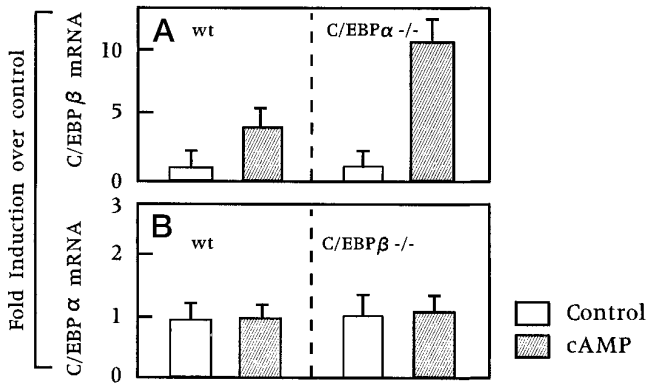


FIG. 7. **Effect of $Bt_2$cAMP on the expression of the genes for C/EBP$\alpha$ and C/EBP$\beta$ in the livers of 20-day fetal mice deficient in the genes for C/EBP$\alpha$ and C/EBP$\beta$.** Mice at 20 days of fetal life that were homozygous for deletions in the genes for C/EBP$\alpha$ and C/EBP$\beta$ were either injected *in utero* with $Bt_2$cAMP (125 mg/kg of body weight; *cAMP*) or not injected (*Control*). Two hours later, the animals were killed; the hepatic RNA was isolated; and the level of C/EBP$\beta$ mRNA was determined in the livers of C/EBP$\alpha^{-}$/_ mice, and that of C/EBP$\alpha$ mRNA was determined in the livers of C/EBP$\beta^{-}$/_ mice. The values for C/EBP$\alpha$ and C/EBP$\beta$ were first standardized relative to the level of actin mRNA and then expressed as -fold induction of C/EBP$\alpha$ or C/EBP$\beta$ mRNA relative to control mice, which were assigned a value of 1. The *bars* represent the mean ± S.E. for four to six mice in each group.

tion of the cyclic nucleotide (Fig. 2). While $Bt_2$cAMP caused an induction of PEPCK gene transcription in the livers of C/EBP$\alpha^{-}$/_ mice, the overall level of induction was only 20% of that noted in the livers of mice with the gene for C/EBP$\alpha$, suggesting that if C/EBP$\beta$ compensates for C/EBP$\alpha$ in the livers of these mice, it is less efficient in altering the rate of transcription of the PEPCK gene.

## DISCUSSION

Glucose homeostasis at birth involves the acute regulation of two pathways involved in glucose production, glycogenolysis, and gluconeogenesis. During the last trimester of pregnancy, the fetal liver synthesizes glycogen, which is mobilized at birth to maintain the level of blood glucose in the neonate (46). The enzymes of gluconeogenesis are present in the mammalian liver before birth, except for PEPCK, which is transcribed for the first time immediately after birth, resulting in the initiation of hepatic gluconeogenesis in the newborn (47). The hormonal factors that coordinate the complex pattern of development of these processes are only partly understood. At birth, there is a dramatic fall in the level of glucose and insulin in the blood and a marked rise in the concentration of glucagon and epinephrine (19). This results in a rapid elevation of the concentration of hepatic cAMP (19), which stimulates glycogenolysis and induces PEPCK gene expression (48). Transcription of the gene for PEPCK can be prematurely induced by the injection of cAMP directly into fetal animals *in utero* (49), whereas the administration of insulin and/or glucose to newborn animals will prevent the initiation of PEPCK gene expression (50). The control of the appearance of glycogen in the liver and the premature induction of PEPCK are developmentally timed. In the rat, both the synthesis of hepatic glycogen (46) and the ability of cAMP to induce PEPCK gene transcription occur at about day 15 of fetal life.[3] This coincides with the initial expression of the genes for C/EBP$\alpha$ and C/EBP$\beta$, both of which have previously been shown to be involved in the transcription of the gene for PEPCK in hepatoma cells in culture (8).

*C/EBP Regulation of PEPCK Gene Transcription in the Liver during Development*—The gene for PEPCK is a target for members of the C/EBP family of transcription factors. The PEPCK promoter contains four C/EBP recognition motifs, including CRE (positions −87 to −74), P3(I) (positions −248 to −230), and P4 (positions −320 to −269) sequences, which bind these transcription factors with varying affinities (Fig. 1) (17, 51–53). Studies using transgenic mice have shown that the P3(I) region, a bona fide C/EBP motif in the PEPCK promoter, is necessary for the specific expression of a transgene in the livers of newborn or adult mice (54). In fact, a mutation in the P3(I) sequence of the PEPCK promoter in the transgene resulted in increased expression in the kidney (54), a tissue in which members of the C/EBP family are poorly expressed (8). In contrast to the liver, the renal expression of the PEPCK gene initiates before birth (55), suggesting its independence from C/EBP. DNA transfection studies using cultured hepatoma cells have shown that C/EBP$\alpha$, C/EBP$\beta$, and DBP stimulate transcription from the PEPCK promoter through P3(I) and P4 sites and the CRE (52). Recently, it has been shown that either C/EBP$\alpha$ or C/EBP$\beta$ synergizes with HNF-1 to stimulate transcription from the PEPCK promoter in hepatoma cells; this synergism requires the presence of P3(I) and P4 sites, but not the CRE (56).

*C/EBP Gene Knockouts Used to Study Hepatic PEPCK Gene Expression*—It is reasonable to assume that transcription of the PEPCK gene in the liver can be regulated by members of the C/EBP family. However, it was not clear which isoform of C/EBP was critical for the hepatic expression of the PEPCK gene until the report of Wang *et al.* (24), which demonstrated a profound disruption of glucose homeostasis in mice homozygous for a deletion in the gene for C/EBP$\alpha$. The genes for glucose-6-phosphatase, PEPCK, and glycogen synthase are not expressed normally in the livers of these mice in the period immediately after birth in the expected manner. Hepatic PEPCK and glucose-6-phosphatase mRNAs were not detectable 2 h after birth; surprisingly, Wang *et al.* (24) found that mRNAs for both enzymes appeared in the liver at 7 and 32 h after birth (the administration of glucose to the pups was required for survival to 32 h). In contrast, the levels of hepatic glycogen synthase mRNA remained virtually undetectable. Thus, C/EBP$\alpha$ is a key factor in the control of PEPCK gene expression in the *immediate* perinatal period, but there are clearly other factors that can induce PEPCK gene transcription

[3] L. Reshef, unpublished observations.

if the animals survive the first few hours after birth.

Mice homozygous for a deletion in the gene for C/EBPβ were initially generated to study the effects of C/EBPβ on the interleukin-6 signaling pathway. Screpanti *et al.* (25) reported that the mice developed a pathology similar to that of animals that overexpress interleukin-6; they have splenomegaly, peripheral lymphadenopathy, enhanced hemopoiesis, and an altered T-helper function. Despite these problems with the immune system, the mice had no *overt* disruption of glucose homeostasis (25, 26). However, both Screpanti *et al.* (25) and Tanaka *et al.* (26) noted a failure to obtain the expected mendelian ratio of mice heterozygous for a deletion in the gene for C/EBPβ, although the appropriate number of C/EBPβ$^{-}$/_ mice were present at 18 days of fetal life (25). In this study, we report that there are two phenotypes noted with C/EBPβ$^{-}$/_ mice. Animals with phenotype A live until ~4–6 months of age and die of problems associated with a severely compromised immune system, whereas the other half of the C/EBPβ$^{-}$/_ mice, those with phenotype B, die within the first hour after birth of profound hypoglycemia. The livers of the animals with phenotype B contain normal levels of glycogen, which is not mobilized appropriately by the administration of $Bt_2$cAMP to the newborn animals. The mice with phenotype B also have no detectable PEPCK mRNA in their livers and are thus unable to synthesize glucose. The injection of $Bt_2$cAMP into these mice 2 h after delivery induced the appearance of PEPCK mRNA, indicating that the gene is responsive to the cyclic nucleotide even in the absence of C/EBPβ. In contrast, the PEPCK gene in the livers of C/EBPα$^{-}$/_ mice will not respond to the cyclic nucleotide when injected 2 h after delivery. These findings clearly demonstrate that C/EBPα, and not C/EBPβ, is required for the response of PEPCK gene transcription to cAMP during the perinatal period.

The reason why there are two different phenotypes noted with C/EBPβ$^{-}$/_ mice is not clear. We assume that there are factors produced in the mice with phenotype A that allow them to transcribe genes critical for survival during the perinatal period. These "modifier genes" are expressed as a result of the genetic background of the mice, which are not inbred. This possibility is supported by preliminary experiments in which mice homozygous for a deletion in the gene for C/EBPβ were backcrossed with C57BL6 mice; no C/EBPβ$^{-}$/_ offspring from these matings survived after birth.[4] One simple explanation for this result is that the gene(s) for other member(s) of the C/EBP family of transcription factors are up-regulated in mice with the A phenotype, permitting their survival through the perinatal period. However, there is no apparent up-regulation of the expression of the gene for C/EBPα or C/EBPδ [4] in the livers of C/EBPβ$^{-}$/_ mice; the level of expression of the genes for the other members of the C/EBP family has not yet been investigated in detail in these mice.

*Regulation of the Development of C/EBP Isoforms in the Liver*—The pattern of development of some of the C/EBP isoforms in the liver has been determined. Specific C/EBP isoforms are induced differentially during the course of development in rodent species. The expression of the gene for C/EBPα in the liver of the mouse begins after day 12 of fetal life, and it accumulates at low levels until birth, when there is a slight increase in the levels of mRNA for both transcription factors (8). The concentrations of both C/EBPβ and C/EBPδ are also low in the fetal liver and rise markedly after birth (57). At ~2–3 weeks after birth, the concentrations of all three of these C/EBP isoforms in the liver increase dramatically to adult levels (57, 58). In contrast to the other forms of C/EBP, DBP accumulates to significant levels only in the livers of adult animals (10).

Our results further suggest that insulin differentially regulates C/EBPα gene expression in the livers of fetal rats since a reduced level of insulin stimulates the transcription of the C/EBPα gene. This effect is not mediated via cAMP, which, by itself, has no effect on the expression of the C/EBPα gene. This conclusion is reinforced by our previous findings that in fetuses, insulin deficiency does not affect the hepatic concentration of cAMP (44). Therefore, insulin deficiency initiates a signal transduction pathway that is independent of that initiated by cAMP. It has recently been shown that insulin selectively decreases C/EBPα gene expression in cultured adipocytes at both the transcriptional and post-transcriptional levels (13). Thus, the expression of the gene for C/EBPα is repressed by insulin both in the fetal liver and in adipocytes in culture.

There is an accumulation of both C/EBPα and C/EBPβ in the livers of newborn rats at the same time that there is an abrupt decline in the concentration of insulin in the blood and a concomitant rise in the level of hepatic cAMP (19). It is thus likely that the reciprocal hormonal environment prevailing in the fetus, *i.e.* high blood insulin levels and low hepatic cAMP, ensures low concentrations of both C/EBPα and C/EBPβ. The injection of $Bt_2$cAMP or the experimental induction of insulin deficiency allowed us to observe that these two treatments initiate processes that operate via independent signal transduction pathways, which, in turn, differentially regulate the expression of the genes for the two C/EBP transcription factors. These pathways are likely to be of fundamental importance in determining the expression of a number of genes during the perinatal period, in particular, the genes that are targets for the two C/EBP transcription factors.

*Conclusions and Some Speculations on the Relative Roles of C/EBP Isoforms in the Regulation of PEPCK Gene Transcription*—While it is now established that the interaction of C/EBPα and C/EBPβ is critical for controlling PEPCK gene expression in the liver during the perinatal period, there are several major questions that arise from this study that will require further detailed investigation. It seems unlikely that the acute effects of insulin deficiency and cAMP on the expression of the genes for C/EBPα and C/EBPβ, respectively, are required for the transcriptional response of the PEPCK gene. We have shown previously that the administration of $Bt_2$cAMP to fetal rats *in utero* results in a rapid induction of transcription of the PEPCK gene; the kinetics of this effect are too rapid to be explained by the prior induction of transcription of the gene for C/EBPβ. This study supports this suggestion since C/EBPβ$^{-}$/_ mice with phenotype A survive the perinatal period with normal levels of PEPCK; even mice with phenotype B can respond to the administration of $Bt_2$cAMP with an induction of PEPCK gene expression in the liver, suggesting that C/EBPβ is not required for the transcriptional response of the PEPCK gene in the liver. If this is the case, then why is the expression of the gene for C/EBPβ induced so markedly by the administration of cAMP? It is possible that C/EBPβ is required to heterodimerize with C/EBPα during the perinatal period so that the induction of its transcription by $Bt_2$cAMP serves the key function of maintaining an appropriate level of this protein in the liver. Alternatively, C/EBPβ may be required to induce the expression of a gene coding for another critical transcription factor, which will, in turn, maintain the level of expression of the PEPCK gene.

Another question that arises from studies with C/EBPα$^{-}$/_ mice involves the temporal expression of the PEPCK gene during the immediate perinatal period. Wang *et al.* (24) noted that there were negligible levels of PEPCK and glucose-6-

[4] V. Poli, unpublished observations.

phosphatase mRNAs in the livers of C/EBP$\alpha^{-}$/_ mice at 2 h after birth, but that the wild-type level of both mRNAs was present at 7 and 32 h after birth. It is possible that C/EBP$\alpha$ is required for PEPCK gene expression only during the first few hours after birth and that another member of the C/EBP family then becomes critical for the regulation of transcription of the PEPCK gene in the liver. Interestingly, we have not been able to detect a mouse at 19 days of fetal life that is homozygous for a deletion in both genes for C/EBP$\alpha$ and C/EBP$\beta$, suggesting that one of the two genes is critical for the regulation of some key processes during development and that one can substitute for the other in the earlier phases of fetal development.

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REFERENCES

1. Greengard, O. (1970) in *Biochemical Action of Hormones* (Litwack, G., ed) Vol. I, pp. 53–87, Academic Press, New York
2. Powell, D. J., Friedman, J. M., Oulette, A. J., Krauter, K. S., and Darnell, J. E., Jr. (1984) *J. Mol. Biol.* **179,** 21–35
3. De Simone, V., and Cortese, R. (1991) *Curr. Opin. Cell Biol.* **3,** 960–965
4. Crabtree, G. R., Schibler, U., and Scott, M. P. (1992) in *Transcriptional Regulation* (McKnight, S. L., and Yamamoto, K. R., eds) pp. 1063–1102, Cold Spring Harbor Laboratory Press, Plainview, NY
5. Xanthopoulos, K. G., and Mirkovitch, J. (1993) *Eur. J. Biochem.* **216,** 353–360
6. Zaret, K. S. (1993) in *Hepatic Transport and Bile Secretion: Physiology and Pathophysiology* (Tavloni, N., and Berk, P. D., eds) pp. 135–143, Raven Press, Ltd., New York
7. Ang, S.-L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J., and Zaret, K. S. (1993) *Development* (*Camb.*) **119,** 1301–1315
8. Berkenmeier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschulz, W. H., and McKnight, S. L. (1989) *Genes Dev.* **3,** 1146–1156
9. Descombes, P., and Schibler, U. (1991) *Cell* **67,** 569–579
10. Mueller, C. R., Maire, P., and Schibler, U. (1990) *Cell* **61,** 279–291
11. McKnight, S. L., Lane, M. D., and Glueckshon-Waelch, S. (1989) *Genes Dev.* **3,** 2021–2024
12. MacDougald, O. A., Cornelius, P., Lin, F.-T., Chen, S. S., and Lane, M. D. (1994) *J. Biol. Chem.* **269,** 19041–19047
13. MacDougald, O. A., Cornelius, P., Liu, R., and Lane, M. D. (1995) *J. Biol. Chem.* **270,** 647–654
14. Mischoulon, D., Rana, B., Bucher, N. L., and Farmer, S. R. (1992) *Mol. Cell. Biol.* **12,** 2553–2560
15. Rana, B., Mischoulson, D., Xie, Y., Bucher, N. L. R., and Farmer, S. R. (1994) *Mol. Cell. Biol.* **14,** 5858–5869
16. Mencher, D., Cohen, H., Benvenisty, N., Meyuhas, O., and Reshef, L. (1984) *Eur. J. Biochem.* **141,** 199–203
17. Trus, M., Benvenisty, N., Cohen, H., and Reshef, L. (1990) *Mol. Cell. Biol.* **10,** 2418–2422
18. Benvenisty, N., and Reshef, L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 1132–1136
19. Girard, J. R., Cuendet, G. S., Marliss, E. B., Kervran, A., Rieutort, M., and Assan, R. (1973) *J. Clin. Invest.* **52,** 3190–3200
20. Hanson, R. W., Reshef, L., and Ballard, J. (1975) *Fed. Proc.* **34,** 166–171
21. Mencher, D., and Reshef, L. (1979) *Eur. J. Biochem.* **94,** 581–589
22. Roesler, W. J., Vandenbark, G. R., and Hanson, R. W. (1989) *J. Biol. Chem.* **264,** 9657–9664
23. Gurney, A. L., Park, E. A., Giralt, M., Liu, J., and Hanson, R. W. (1992) *J. Biol. Chem.* **267,** 18133–18139
24. Wang, N. D., Finegold, M., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995) *Science* **269,** 1108–1112
25. Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, D., Sellitto, C., Scarpa, S., Bellavia, D., Lattanzio, G., Bistoni, F., Frati, L., Cortese, R., Gulino, A., Ciliberto, G., Costantini, F., and Poli, V. (1995) *EMBO J.* **14,** 1932–1941
26. Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, N., Shirufuji, N., Fujiwara, H., Suematsu, S., Yoshida, N., and Kishimoto, T. (1995) *Cell* **80,** 353–361
27. Loose, D., Shaw, P., Krauter, K. S., Robinson, C., Englard, S., Hanson, R. W., and Gluechsohn-Waelsch, S. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5184–5188
28. Yoo-Warren, H., Monahan, J. E., Short, J., Short, H., Bruzel, A., Wynshaw-Boris, A., Meisner, H. M., Samols, D., and Hanson, R. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3656–3660
29. Friedman, A. D., Landschultz, W. H., and McKnight, S. L. (1989) *Genes Dev.* **3,** 767–776
30. Poli, V., Mancini, F. P., and Cortese, R. (1990) *Cell* **63,** 643–653
31. Sherman, L., Dafni, J., Lieman-Hurwitz, J., and Groner, Y. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 5465–5468
32. Kioussis, D., Hamilton, R., Hanson, R. W., Tilghman, S. M., and Taylor, J. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4370–4374
33. Gunning, P., Ponte, P., Kedes, L., Hickey, R. J., and Skoultchi, A. I. (1984) *Cell* **36,** 709–715
34. Eisenberger, C. L., Nechushtan, H., Cohen, H., Shanti, M., and Reshef, L. (1992) *Mol. Cell. Biol.* **12,** 1396–1403
35. Chirgwin, J. M., Prezybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–6000
36. Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 1408–1412
37. Meisner, H., Loose, D. S., and Hanson, R. W. (1985) *Biochemistry* **24,** 421–425
38. Schibler, U., Hagenbuchle, O., Wellauer, P. K., and Pittet, A. C. (1983) *Cell* **33,** 501–508
39. Groudine, M., Peretz, M., and Weintraub, H. (1981) *Mol. Cell. Biol.* **1,** 281–288
40. Aloni, R., Peleg, D., and Meyuhas, O. (1992) *Mol. Cell. Biol.* **12,** 2203–2212
41. Gorski, K., Carneino, M., and Schibler, U. (1986) *Cell* **47,** 767–776
42. Towbin, H., Stahlin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
43. Cascio, F., and Zaret, K. S. (1991) *Development* (*Camb.*) **113,** 217–225
44. Mencher, D., Shouval, D., and Reshef, L. (1979) *Eur. J. Biochem.* **102,** 489–495
45. Park, E. A., Gurney, A. L., Nizielski, S. E., Hakimi, P., Cao, Z., Moorman, A., and Hanson, R. W. (1993) *J. Biol. Chem.* **268,** 613–619
46. Ballard, F. J. (1970) in *Physiology of the Perinatal Period* (Stave, U., ed) Vol. 1, pp. 417–440, Appleton-Century-Crofts, New York
47. Ballard, F. J., and Hanson, R. W. (1967) *Biochem. J.* **104,** 866–871
48. Hopgood, M. F., Ballard, F. J., Reshef, L., and Hanson, R. W. (1973) *Biochem. J.* **134,** 445–453
49. Garcia-Ruiz, J. P., Ingram, R., and Hanson, R. W. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 4189–4193
50. Philippidis, H., and Ballard, F. J. (1969) *Biochem. J.* **113,** 651–657
51. Park, E. A., Roesler, W. J., Liu, J., Klemm, D. J., Gurney, A. L., Thatcher, J., Shuman, J., Friedman, A., and Hanson, R. W. (1990) *Mol. Cell. Biol.* **10,** 6264–6272
52. Park, K., and Kim, K.-H. (1993) *J. Biol. Chem.* **268,** 17811–17819
53. Roesler, W. J., Simard, J., Graham, J. G., and McFie, P. J. (1994) *J. Biol. Chem.* **269,** 14276–14283
54. Patel, Y. M., Yun, J. S., Liu, J., McGrane, M. M., and Hanson, R. W. (1994) *J. Biol. Chem.* **269,** 5619–5628
55. Zorzoli, A., Turkenkopf, I. J., and Mueller, V. L. (1969) *Biochem. J.* **111,** 181–185
56. Yanuka-Kashles, O., Cohen, H., Trus, M., Aran, A., Benvenisty, N., and Reshef, L. (1994) *Mol. Cell. Biol.* **14,** 7124–7133
57. Diehl, A. M., Michaelson, P., and Yang, Q. Y. (1994) *Gastroenterology* **106,** 1625–1637
58. Nagy, P., Bisgaard, H. C., and Thorgeirsson, S. S. (1994) *J. Cell Biol.* **126,** 223–233
59. Giralt, M., Park, A. E., Gurney, A., Liu, J. S., Hakimi, P., and Hanson, R. W. (1991) *J. Biol. Chem.* **266,** 21991–21996
60. Imai, E., Stromstedt, P. E., Quinn, P. G., Carlstedt-Duke, J., Gustaffson, J. A., and Granner, D. K. (1990) *Mol. Cell. Biol.* **10,** 4712–4719
61. O'Brien, R. M., Lucas, P. C., Forest, C. D., Magnunson, M. A., and Granner, D. K. (1990) *Science* **249,** 533–537