J. Biochem. 119, 524-532 (1996)

Induction of the C/EBPβ Gene by Dexamethasone and Glucagon in Primary-Cultured Rat Hepatocytes[1]

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Received for publication, October 16, 1995

The synthetic glucocorticoid, dexamethasone, and glucagon cooperatively elevated the level of mRNA for the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) in primary-cultured rat hepatocytes. In response to dexamethasone and/or glucagon, C/EBPβ mRNA started to increase as early as at 30 min, reached a maximum within 2 h, and then gradually decreased. The administration of cycloheximide, a protein synthesis inhibitor, led rather to an increase in C/EBPβ mRNA, which suggested that a labile negative protein factor(s) is involved in regulation of the C/EBPβ mRNA level. Cycloheximide further augmented the increases in C/EBPβ mRNA by dexamethasone and/or glucagon. Therefore, C/EBPβ mRNA accumulation in response to these hormones is apparently independent of ongoing protein synthesis. The elevation of the C/EBPβ mRNA level by these hormones was accounted for by increases in the rate of transcription of the C/EBPβ gene, as deduced on nuclear run-on analysis. Gel mobility shift analysis revealed that the DNA-binding activity of C/EBPβ was increased cooperatively by dexamethasone and glucagon. These results suggest that the C/EBPβ gene is primarily induced by glucocorticoids and/or glucagon and that the accumulated C/EBPβ protein is then involved in secondary activation of target genes in response to these hormones in the liver.

Key words: C/EBPβ, dexamethasone, glucagon, liver, primary hepatocytes.

A number of genes involved in the exhibition of liver-specific traits such as gluconeogenesis and urea biosynthesis are regulated hormonally (for recent reviews, see Refs. *1-4*). In the course of the development of mammals from the late fetal to neonatal period, a set of liver-selective genes is induced. This induction can be triggered by glucocorticoids and/or glucagon (or analogs of cAMP, a second messenger of glucagon). After birth, these genes are also under the positive control of glucocorticoids and/or glucagon, which seem to mediate the effects of environmental fluctuations such as dietary changes (*1-4*). The responses of gene transcription to hormones can be categorized into two classes: primary and secondary. The primary responses occur rapidly without the requirement of new protein synthesis, and result from modification of preexisting transcriptional regulators. The secondary responses are delayed in the timing of onset and are sensitive to protein synthesis inhibitors, suggesting the involvement of a transcriptional regulator(s) newly synthesized through an earlier response. A typical model of primary and secondary responses is provided by an insect steroid hormone, ecdysone, in *Drosophila*. On the onset of metamorphosis from the larval to adult stage, ecdysone triggers a cascade of gene activations, some of which can be monitored in the salivary gland as polytene chromosome puffs (reviewed in Refs. *5* and *6*). Less than ten puffs appear rapidly in response to ecdysone, whereas more than 100 late puffs are induced by gene products of earlier processes. A secondary response that has also been noted is the action of glucocorticoids in mammalian tissues including the liver. For example, the genes for hepatic α_{2u}-globulin (*7*) and its isoform (*8, 9*), and ornithine cycle enzymes such as carbamylphosphate synthetase, argininosuccinate lyase and arginase (*10*) are induced by glucocorticoids with delayed onset depending on *de novo* protein synthesis. As for glucagon, while it induces a number of genes rapidly, repression of the induction by protein synthesis inhibitors has been noted for several genes such as the serine aminotransferase gene (*11*) and ornithine cycle enzyme genes (*10*). This suggests the possibility that the induction of these genes by glucagon is triggered in a primary manner and is sustained in a secondary manner. The transcriptional regulator(s) that is induced primarily by glucocorticoids or glucagon and then activates the target genes has remained to be identified.

CCAAT/enhancer binding protein (C/EBP) β (*12*), also known as NF-IL6 (*13*), IL6-DBP (*14*), LAP (*15*), AGP/EBP (*16*), and CRP2 (*17*), is a transcription factor of the basic region/leucine zipper type and is a member of the C/EBP family, the prototype of which is a liver-selective

[1] This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (to H.M., K.T., M.M., and M.T.), and grants from the Ministry of Health and Welfare of Japan (to H.M., K.T., and M.M.).

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Abbreviations: C/EBP, CCAAT/enhancer binding protein; HNF-3β, hepatocyte nuclear factor-3β; GR, glucocorticoid receptor; CREB, cAMP-responsive element-binding protein; bp, base pair(s).

transcription factor, C/EBPα (*12, 18*). While C/EBPβ is also abundant in the liver and plays a role in the liver-selective transcription of target genes (*15, 19, 20*), it seems to be involved in various cellular processes in a variety of tissues, including differentiation and activation of myelomonocytic cells (for a review, see introduction of Ref. *21*), adipocyte differentiation (*12, 22*), and the acute-phase respose in the liver (*13, 14, 16*). We here investigated the possibility that this factor mediates the secondary response to glucocorticoids and/or glucagon, themselves having been induced primarily. We found that the C/EBPβ gene is induced cooperatively by dexamethasone and glucagon in primary-cultured rat hepatocytes. During the course of this study, elevation of the C/EBPβ mRNA level by dexamethasone in a hepatoma cell line (*23, 24*) and by a cAMP analog in the liver of a whole rat (*25*) was briefly reported. The present study demonstrates that dexamethasone and glucagon, and their combination cooperatively induce the C/EBPβ gene in a primary manner, and suggests that C/EBPβ is involved in secondary induction of target genes in response to these hormones.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Hepatocytes—Parenchymal hepatocytes from male Wistar rats (6-9 weeks) were isolated by *in situ* perfusion of their livers with collagenase as described (*26, 27*). 3×10^6 cells were seeded into collagen-coated plastic dishes (Corning, 100ϕ) with 10 ml of Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 100 μg/ml streptomycin. The medium was changed 24 h after plating.

Isolation and Blot Analysis of RNA—Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (*28*). The RNA (5 μg per lane) was electrophoresed in denaturing formaldehyde-agarose (1%) gels. After visualizing 28S and 18S rRNAs by ethidium bromide staining to check the integrity of RNA samples and equal loading, the RNA was transferred onto nylon membranes. Hybridization was performed using DNA probes ^{32}P-labeled by the random priming method. Radioactive bands were visualized by autoradiography on X-ray films and the radioactivity was measured with a Bio-Image Analyzer BAS2000 (Fuji Photo Film, Tokyo). RNA was usually prepared from three or more independent cultures. Relative mRNA levels are shown as mean values with standard errors. The following DNA probes were used: mouse C/EBPα, the 415-bp *Sma*I fragment of the mouse gene (*20, 29*); mouse C/EBPβ, the 390-bp *Nco*I fragment of the mouse gene (*12, 16, 20*); rat arginase, the ~850-bp *Eco*RI-*Eco*RV cDNA fragment of pARGr-2 (*30*).

Nuclear Run-On Analysis—Nuclei from primary-cultured hepatocytes were isolated by the detergent treatment procedure described (*31*). Nuclei derived from $1.0\text{-}1.5\times10^7$ cells were suspended in 100 μl of a buffer containing 50 mM Tris-HCl (pH 8.3), 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA, and stored at −80°C. Run-on transcription was started by mixing the thawed nuclear suspension with 150 μl of a solution containing 10 mM Tris-HCl (pH 8.0), 300 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 1 mM CTP, 1 mM GTP, and 60 μCi [α-^{32}P]UTP (3,000 Ci/mmol), and allowed to proceed for 30 min with shaking at 30°C. Labeled RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (*28*) as follows. The reaction was stopped by adding 300 μl of a solution containing 5.3 M guanidinium thiocyanate, 33 mM sodium citrate, 0.67% sarcosyl, and 0.1 M β-mercaptoethanol, followed by mixing with 50 μl of 2 M sodium acetate (pH 4.0). After extraction with 500 μl of water-saturated phenol and 200 μl of chloroform, RNA in the resultant aqueous phase was precipitated with 600 μl of isopropanol at room temperature for 10 min. The precipitate was collected by centrifugation, washed with 75% ethanol, and suspended in 100 μl of water.

To increase the hybridization specificity and efficiency, antisense strand RNAs synthesized *in vitro* with SP6 RNA polymerase were used as probes. The template DNA for the C/EBPβ probe corresponds to the ~850-bp *Pst*I-*Eco*RI segment of the mouse C/EBPβ gene (*20*). This segment covers the region encoding the C-terminal 31 amino acid residues and the 3′ noncoding region, in which the nucleotide sequences of the mouse (*12, 16*) and rat (*15*) C/EBPβ genes are well conserved. The ~850-bp segment was inserted into the *Xho*I and *Bam*HI sites of the plasmid pcDNAII (Invitrogen, Netherlands) after manipulating both ends. The resultant plasmid, named pcDNAII-C/EBPβ-2, was linearized by *Xho*I digestion and then subjected to *in vitro* transcription with SP6 RNA polymerase. As a control, the antisense strand RNA of rat hepatocyte nuclear factor-3β (HNF-3β) was used; the reverse transcription/polymerase chain reaction product that was derived from rat liver RNA and that corresponds to nucleotide positions 182 to 1576 of the published HNF-3β cDNA sequence (*32*) was subcloned into the *Eco*RV site of pcDNAII, and the resultant plasmid, named pcDNAII-HNF-3β, was cut with *Xho*I and processed for transcription. Antisense strand RNAs (2 μg) were separated by electrophoresis in denaturing formaldehyde-agarose gels and blotted onto nylon membranes.

Membrane strips harboring blotted RNAs were subjected to hybridization at 68°C for 16 h in 2 ml of a buffer containing 50% formamide, 5×Denhardt's solution, 0.75 M NaCl, 50 mM NaH_2PO_4 (pH 7.4), 5 mM EDTA, 0.1% SDS, 0.1 mg/ml denatured salmon testis DNA, and 3.5×10^6 dpm of ^{32}P-labeled RNA. After washing at 68°C in a solution containing 15 mM NaCl, 1.5 mM sodium citrate, and 0.1% SDS, the hybridized bands were visualized and the radioactivity was measured with a Bio-Image Analyzer.

Gel Mobility-Shift Assay—Nuclear extracts of hepatocytes were prepared by the mini-scale detergent treatment procedure described (*33*). Double-stranded synthetic oligonucleotides were 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and used as probes. The binding reaction was carried out on ice for 30 min in 10 μl of a mixture containing 25 mM Hepes-KOH (pH 7.6), 50 mM KCl, 1 mM EDTA, 0.5 mM spermidine, 0.6 mM dithiothreitol, 12% glycerol, 5 μg of poly(dI-dC), 1 fmol of the probe (about 2×10^4 dpm), and 5 μg of nuclear extract protein. In the antibody supershift assay, the affinity-purified rabbit polyclonal antibody (1 μg in 1 μl) against C/EBPα or C/EBPβ purchased from Santa Cruz Biotechnology (Santa Cruz) was added to the binding mixture halfway through the reaction (at 15 min). After incubation, 2.5 μl of 20% Ficoll was added, and then samples were loaded onto 5% polyacrylamide gels made in a buffer containing 67 mM Tris, 67 mM boric acid, and 2 mM

EDTA. Electrophoresis was performed at 10 V/cm for 2 h at room temperature. The gels were then dried and autoradiographed.

RESULTS

Effects of Dexamethasone and Glucagon on the C/EBPβ mRNA Level in Primary-Cultured Hepatocytes—Rat hepatocytes were isolated by collagenase perfusion and cultured on collagen-coated dishes in a medium containing 5% fetal bovine serum. Blot analysis of RNA extracted every 24 h during the culture revealed that the mRNAs for liver-enriched C/EBP family members, C/EBPα and C/EBPβ, remained almost unchanged from the start of the culture up to 120 h (data not shown). The following experiments were thus performed between 24 and 72 h of the culture.

To examine the effects of glucocorticoids and/or glucagon on expression of the genes for C/EBPα and C/EBPβ, we added these hormones separately or in combination to primary-cultured cells twice, *i.e.* 24 and 6 h prior to harvesting, and then carried out RNA blot analysis (Fig. 1). The level of mRNA for C/EBPα was little changed by a synthetic glucocorticoid, dexamethasone, or glucagon, or their combination. On the other hand, C/EBPβ mRNA increased in response to dexamethasone and glucagon, 2.0- and 1.6-fold, respectively. The combination of these hormones led to a 2.9-fold increase, which can be taken as either an additive or synergistic increase, quantitatively.

As a positive control for the effects of the hormones, we also measured the level of mRNA for an ornithine cycle enzyme, arginase, the gene of which is synergistically induced by glucocorticoids and glucagon (or its second messenger analog, dibutyryl cAMP) (*10*). The arginase mRNA increased in response to these hormones: 6.7-fold with dexamethasone, 4.2-fold with glucagon, and 62-fold with their combination. The extent of this mRNA induction in response to the hormones was comparable to findings previously reported (*10*) and our observation (Takiguchi,

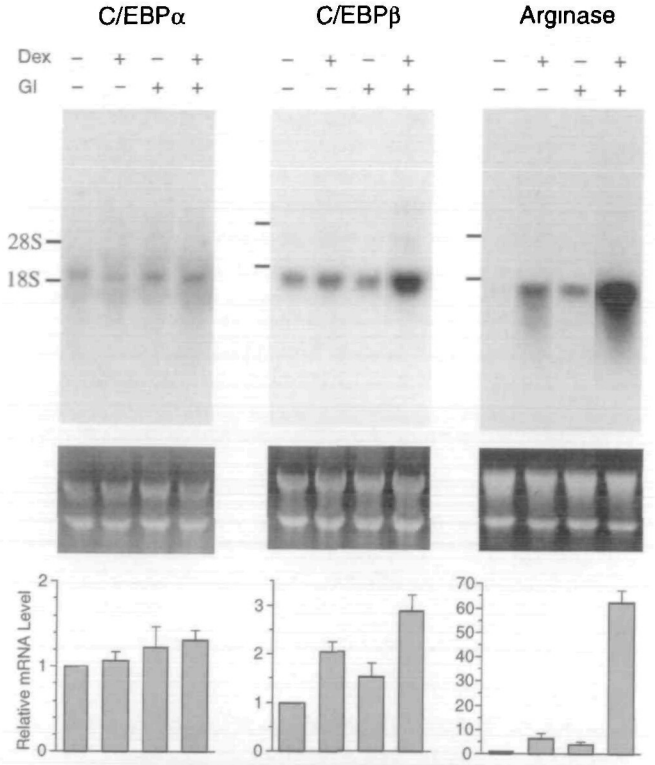

Fig. 1. **Increase in C/EBPβ mRNA by dexamethasone and/or glucagon in primary-cultured rat hepatocytes.** Dexamethasone (10^{-6} M, Dex) and/or glucagon (3×10^{-8} M, Gl) were added or not added to the culture medium of primary hepatocytes twice, *i.e.* 24 and 6 h prior to harvesting of the cells, and RNA blot analyses for C/EBPα, C/EBPβ, and arginase were carried out. Top. Representative autoradiographs are shown. The positions of 28S and 18S rRNA are indicated. Middle. rRNA staining profiles are shown. Bottom. The radioactivity of the hybridized bands was measured and is shown relative to the level obtained without hormone addition. Bars represent means + standard errors.

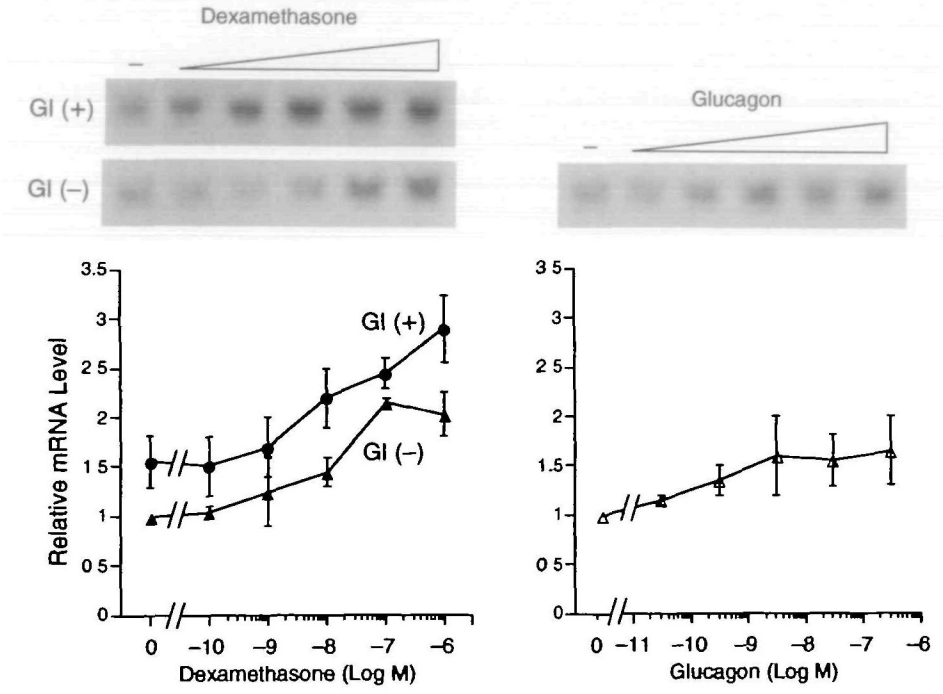

Fig. 2. **Dose-dependent increase in C/EBPβ mRNA by dexamethasone and glucagon.** Various concentrations of dexamethasone (left panel) and glucagon (right panel) were added or not added to the culture medium of primary hepatocytes twice, *i.e.* 24 and 6 h prior to harvesting of the cells, and RNA blot analysis for C/EBPβ was carried out. In the left panel, Gl (+) and Gl (−) denote the addition and omission, respectively, of 3×10^{-8} M glucagon simultaneously with dexamethasone. The radioactivity of the hybridized bands is shown relative to the level obtained without hormone addition. Individual values are means ± standard errors. At the top, representative autoradiographs are shown.

M., unpublished results), where hepatocytes were cultured in a serum-free medium. Therefore, the effects of the hormones derived from serum were almost negligible under the present experimental conditions.

Characterization of Hormonal Induction of C/EBPβ mRNA—The dependence of C/EBPβ mRNA induction on the concentrations of dexamethasone and glucagon was then examined (Fig. 2). The mRNA level increased in response to dexamethasone in a dose-dependent manner (left panel). The half-maximal response was obtained at a concentration around 10^{-8} M, this being in agreement with values reported for other responses mediated by the dexamethasone-glucocorticoid receptor (GR) complex (*34-36*). In the presence of 3×10^{-8} M glucagon, the response curve against the concentration of dexamethasone shifted upward about 1.5-fold. Glucagon also raised the level of C/EBPβ mRNA in a dose-dependent manner (right panel), the half-maximal response being observed at a concentration around 3×10^{-10} M, which is within the physiological glucagon concentration range in the rat portal vein (*37*).

The time course of changes in the C/EBPβ mRNA level by dexamethasone and/or glucagon was also monitored (Fig. 3). In response to dexamethasone, C/EBPβ mRNA increased slightly as early as by 30 min, reached a maximum by 2 h, and then gradually decreased to a control level by 48 h. With glucagon, a similar time course pattern was observed, except that the effect continued for up to 48 h. With the combination of dexamethasone and glucagon, the rapid increase at 30 min was more prominent than that seen with each hormone alone, followed by a likewise magnified maximum at 2 h and a succeeding gradual decrease. The rapid increases in C/EBPβ mRNA in response to dexamethasone and/or glucagon suggest that these agents directly elevate the level of C/EBPβ mRNA independent of ongoing protein synthesis.

Effect of a Protein Synthesis Inhibitor on the Increase in C/EBPβ mRNA—To confirm that the hormonal responses of the C/EBPβ mRNA level are primary, *i.e.* they require no prior protein synthesis, we examined the effect of a protein synthesis inhibitor, cycloheximide (Fig. 4). As a positive control, we measured the changes in the level of arginase mRNA (Fig. 4B), the induction of which by dexamethasone was shown to be secondary, *i.e.* being mediated by a newly synthesized protein factor(s) (*10*). An

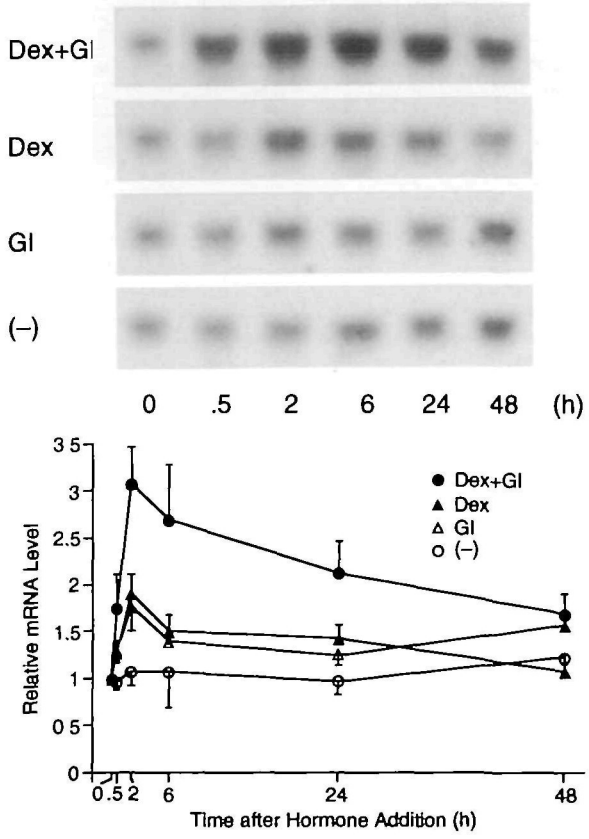

Fig. 3. **Time course of increase in C/EBPβ mRNA by dexamethasone and/or glucagon.** Dexamethasone (10^{-6} M, Dex) and/or glucagon (3×10^{-8} M, Gl) were added or not added to the culture medium of primary hepatocytes, and RNA blot analysis for C/EBPβ was carried out after indicated times. The radioactivity of the hybridized bands is shown relative to the level obtained at the start point. Individual values are means + or − standard errors. At the top, representative autoradiographs are shown.

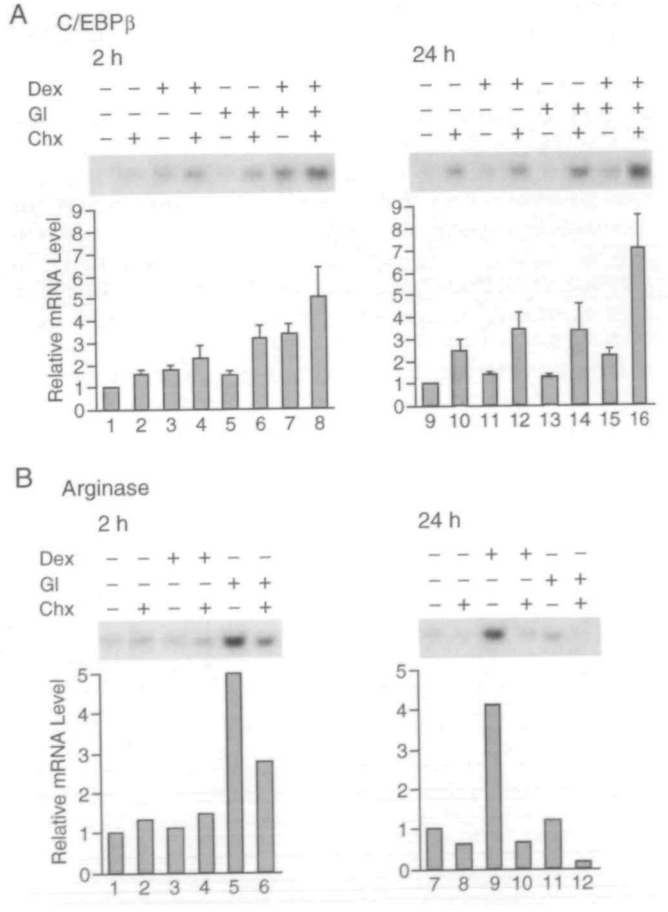

Fig 4. **Effects of cycloheximide on dexamethasone- and/or glucagon-induced C/EBPβ mRNA.** RNA blot analyses for C/EBPβ (A) and arginase (B) were carried out, after primary hepatocytes had been treated or not treated for 2 or 24 h with 10^{-6} M dexamethasone (Dex) and/or 3×10^{-8} M glucagon (Gl), and simultaneously with 2 μg/ml of cycloheximide (Chx) where indicated. The radioactivity of the hybridized bands is shown relative to the level obtained without addition of the agents. Above the histograms, representative autoradiographs are shown. In (A), bars represent means+standard errors In (B), the results of one representative experiment are shown.

increase in arginase mRNA was not evident at 2 h after the addition of dexamethasone but was prominent at 24 h. This delayed accumulation of arginase mRNA was completely repressed by cycloheximide. On the other hand, glucagon increased arginase mRNA within 2 h. This rapid increase was only partially repressed by cycloheximide. Apparently, *de novo* protein synthesis is not essential for glucagon to trigger the arginase mRNA induction, although a labile preexisting protein(s) or a newly synthesized protein(s) may possibly further augment the induction. Thus, cycloheximide treatment under the present conditions successfully distinguished the primary response to glucagon and the secondary response to dexamethasone for the induction of arginase mRNA.

C/EBPβ mRNA was increased 1.6-fold by treatment with cycloheximide alone for 2 h (Fig. 4A). A phenomenon of this type is known as superinduction and suggests that a labile negative protein factor(s) is involved in regulation of the C/EBPβ mRNA level. The increase in C/EBPβ mRNA by dexamethasone was further augmented by cycloheximide. Therefore, C/EBPβ mRNA accumulation in response to dexamethasone does not appear to require ongoing protein synthesis. The increase in C/EBPβ mRNA by glucagon was also augmented by cycloheximide. In accordance with these results, C/EBPβ mRNA accumulation in response to dexamethasone plus glucagon was further increased by cycloheximide. Within 24 h, the effect of cycloheximide was augmented, there being an about 2.5-fold increase in C/EBPβ mRNA. C/EBPβ mRNA accumulation in response to dexamethasone, glucagon and both was further increased by cycloheximide. In conclusion, the increase in C/EBPβ mRNA by dexamethasone, as well as by glucagon, is apparently independent of ongoing protein synthesis.

Effects of Dexamethasone and Glucagon on Transcription of the C/EBPβ Gene—To determine whether the increases in C/EBPβ mRNA by the hormones are caused transcriptionally or post-transcriptionally, the rate of transcription was measured by means of a run-on assay, using nuclei isolated at 2 and 6 h after stimulation with dexamethasone and/or glucagon (Fig. 5) To increase the specificity and sensitivity for the detection of transcripts, antisense RNAs synthesized from cloned genes were blotted onto membranes and used as probes. As a standard, the rate of transcription of the gene for HNF-3β, the mRNA level of which was little changed by either hormone (data not shown), was measured, and used to normalize the hybridization efficiency. Within 2 h of stimulation, dexamethasone caused a 1.6-fold increase in the rate of transcription of the C/EBPβ gene, and glucagon caused a 1.5-fold increase, and their combination resulted in a 3.3-fold increase. At 6 h, with dexamethasone or glucagon alone the transcription was almost at the control level, while a 2.0-fold increase was still observed with their combination. It is obvious that dexamethasone and/or glucagon augment transcription of the C/EBPβ gene to an extent comparable to that of elevation of the mRNA level. Therefore, transcriptional induction can account for the increases in C/EBPβ mRNA in response to these hormones.

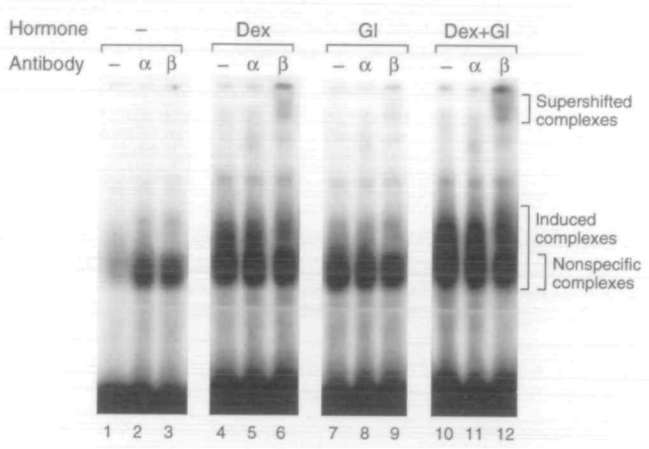

Fig. 6. **Gel shift analysis of changes in DNA-binding activity of C/EBPβ by dexamethasone and/or glucagon.** Nuclear extracts were prepared from primary hepatocytes that had been not treated or treated with 10^{-6} M dexamethasone (Dex) and/or 3×10^{-8} M glucagon (Gl) for 10 h, and subjected to gel shift analysis using the C/EBP site of the Rous sarcoma virus promoter as a probe (*38*). The affinity-purified rabbit polyclonal antibody against C/EBPα (α) or C/EBPβ (β) was added to the binding mixture halfway through the reaction The positions of complexes induced by hormone treatment, supershifted on antibody addition, and nonspecifically augmented on antibody addition are indicated by brackets.

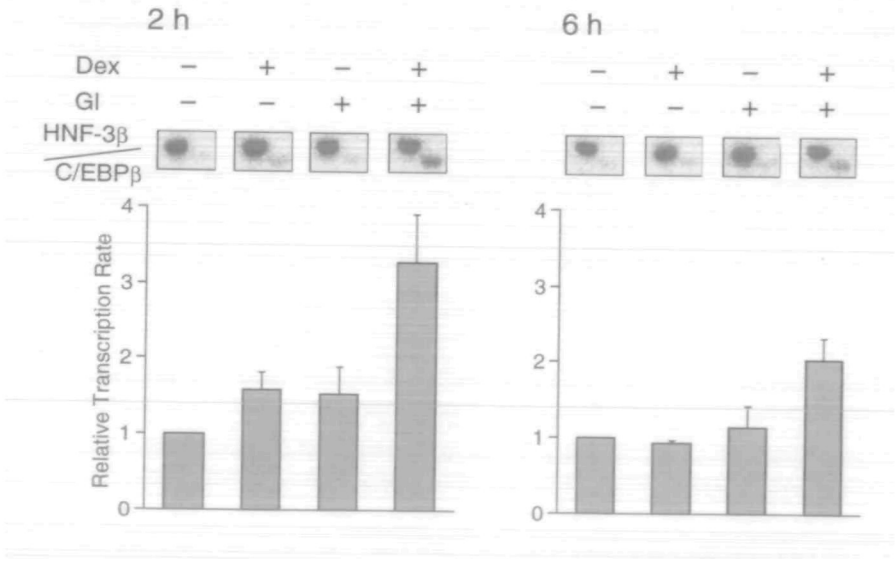

Fig 5. **Nuclear run-on analysis for changes in the rate of transcription of the C/EBPβ gene by dexamethasone and/or glucagon.** Nuclei were isolated from primary hepatocytes that had been not treated or treated with 10^{-6} M dexamethasone (Dex) and/or 3×10^{-8} M glucagon (Gl) for 2 or 6 h, and subjected to run-on assays to determine the rates of transcription of the HNF-3β and C/EBPβ genes simultaneously. The radioactivity of the transcripts hybridized to the C/EBPβ probe was normalized as to the HNF-3β signal, and is shown relative to the level obtained without hormone addition Bars are means+standard errors. Above the histograms, representative autoradiographs are shown.

Effects of Dexamethasone and Glucagon on DNA-Binding Activity of C/EBPβ—To verify that elevation of the C/EBPβ mRNA level leads to an increase in the DNA-binding activity of the protein, gel shift analysis was performed (Fig. 6). Nuclear extracts were prepared from cells treated with dexamethasone and/or glucagon for 10 h, and then subjected to analysis of binding with the C/EBP site of the Rous sarcoma virus promoter (*38*). In response to dexamethasone or glucagon, the intensities of bands corresponding to DNA-protein complexes increased (lanes 4 and 7 compared to lane 1). The combination of both hormones resulted in further augmentation of the complexes (lane 10). This complex formation was competed for by an excess amount of the homologous oligonucleotide (data not shown). To determine if C/EBPβ was included in the hormone-induced complexes, antibody supershift analysis was performed. When added to mixtures containing extracts derived from hormone-untreated cells, antibodies each specific to C/EBPα and C/EBPβ augmented, for an unknown reason, part of the complexes (nonspecific complexes in lanes 2 and 3), which was also augmented by a control antibody (data not shown). The nature of this augmentation was not further investigated here. The C/EBPβ-specific antibody caused supershift of the hormone-induced complexes (lanes 6, 9, and 12) more prominently than was seen with the C/EBPα-specific antibody (lanes 5, 8, and 11). The supershifted bands obtained with the C/EBPβ antibody were more intense in the case of the extracts derived from cells treated with both dexamethasone and glucagon than with either hormone alone. In summary, the DNA-binding activity of C/EBPβ was induced by these hormones, apparently reflecting the changes in the mRNA level.

DISCUSSION

Induction of the C/EBPβ Gene in Response to Dexamethasone and Glucagon—We found that the C/EBPβ gene is induced by dexamethasone and/or glucagon in primary-cultured rat hepatocytes. Previous reports noted that C/EBPβ mRNA is increased by dexamethasone in a hepatoma cell line (*23, 24*), and by a cAMP analog in rat liver (*25*). It was also briefly reported that the administration of both dexamethasone and a cAMP analog causes a slight increase in C/EBPβ mRNA in neonatal mouse liver (*39*). The results of the present study indicate that the effects of dexamethasone and/or glucagon on the induction of C/EBPβ mRNA in hepatocytes are primary: the mRNA accumulation was rapidly induced independent of ongoing protein synthesis through transcriptional activation. The effects of these hormones were cooperative; *i.e.* their combination resulted in a more potent effects, than with each alone, on the transcription rate, mRNA level, and binding activity of C/EBPβ.

Activation of the C/EBPβ gene by dexamethasone appears to be mediated by GR, because of the rapid kinetics of the mRNA induction, similarity in dose dependency to other GR-mediated responses (*34-36*), and insensitivity to cycloheximide.

As for the signal transduction pathway of glucagon, binding of this hormone with its receptor on the cell surface results in increases in the intracellular concentrations of not only cAMP but also 1,2-diacylglycerol and inositol 1,4,5-trisphosphate, and then augments Ca^{2+} mobilization (reviewed in Ref. *40*). Therefore, a number of transcription factors that are targets of these signal transduction pathways are potential candidates for the factor that mediates the effect of glucagon to the C/EBPβ gene. Among them, cAMP-responsive element-binding protein (CREB) (*41, 42*) is one of the best-characterized transcription factors, whose involvement in the cAMP pathway in the liver was reported (*25, 43-45*). Another notable possibility is autostimulation of the C/EBPβ gene by C/EBPβ itself. In rat pheochromocytoma PC12 cells, treatment with forskolin, an inducer of cAMP, causes phosphorylation of C/EBPβ, and stimulates translocation of the protein from the cytosol to the nucleus (*46*). C/EBPβ is also activated by Ca^{2+}/calmodulin-dependent protein kinase II (*47*) and mitogen-activated protein kinases (*48*) through direct phosphorylation. In addition, an unknown protein kinase(s) activated through the protein kinase C pathway causes phosphorylation of C/EBPβ (*49, 50*), which results in enhancement of the transcriptional efficacy without alteration of the affinity of DNA-binding (*49*).

Candidates for Target Genes of Induced C/EBPβ—Induced C/EBPβ may in turn affect the transcription of its target genes, as an activator or a repressor (LIP form in Ref. *51*). Genes affected by glucocorticoids or glucagon in a secondary manner are candidates for targets of induced C/EBPβ. A typical secondary glucocorticoid response follows a delayed time course and is sensitive to protein synthesis inhibitors, as exemplified by α_{2u}-globulin genes (*7-9*), and those of ornithine cycle enzymes such as carbamylphosphate synthetase, argininosuccinate lyase, and arginase (*10*). The glucocorticoid induction of the genes for α_1-acid glycoprotein (*35*), albumin (*52*), and tryptophan oxygenase (*53*) is relatively rapid but remains sensitive to protein synthesis inhibitors. While this type of rapid induction appears to be triggered by the glucocorticoid-GR complex interacting with regulatory regions of the genes, as was demonstrated for the tryptophan oxygenase gene (*54*), the induction may require an additional newly synthesized factor(s) for its further augmentation.

Interestingly, binding of C/EBP family members has been detected in the regulatory regions of some of the above-mentioned genes: the carbamylphosphate synthetase promoter (*55*); the arginase promoter (*56*) and enhancer (*57*); the albumin promoter (*15, 58*) and enhancer (*59*); and the α_1-acid glycoprotein promoter (*16, 60, 61*). Apparently, the presence of the C/EBP-binding site by itself is not sufficient to mediate the secondary glucocorticoid response, as exemplified by the arginase promoter (*57*). On the other hand, C/EBP family members seem to be involved in maximal activation of the α_1-acid glycoprotein promoter by glucocorticoids (*60-62*). The C/EBP site in the arginase enhancer (*57*) was recently shown to be capable of mediating a glucocorticoid response (Gotoh, T., unpublished results). Thus, the arginase gene is a good candidate for the target of C/EBPβ induced by glucocorticoids.

As for the glucagon pathway, while ornithine cycle enzyme genes are rapidly induced by a cAMP analog, the induction is partly sensitive to cycloheximide (*10*), thereby suggesting that both primary and secondary elements are involved in this induction. As mentioned above, the regulatory regions of genes for ornithine cycle enzymes such as

carbamylphosphate synthetase (*55*) and arginase (*56, 57*) are bound by C/EBP family members, but the involvement of these binding sites in the glucagon response was not demonstrated. The cAMP-responsive element in the phosphoenolpyruvate carboxykinase promoter binds with C/EBP family members as well as CREB (*25, 45, 63*). C/EBPβ was suggested to be involved (*25*) in the long-term sustained stimulation of the carboxykinase gene in response to cAMP after the initial strong stimulation (*64*). This view is supported by our present finding that glucagon acts directly on hepatocytes and rapidly increases C/EBPβ mRNA.

C/EBPβ Induction and Other Aspects of the C/EBPβ-Mediated Regulation in Response to Glucocorticoids and Glucagon—In addition to the regulation through transcriptional induction of the gene for C/EBPβ, this factor seems to be involved in several other aspects of transcriptional regulation by glucocorticoids and glucagon. In the promoter region of the α_1-acid glycoprotein gene, there are at least one GR-binding site (*65, 66*) and two C/EBPβ-binding sites (*16, 60, 61*). The GR site and one of the two C/EBPβ sites function cooperatively in the glucocorticoid response (*60, 61*), possibly through the protein-protein interaction between C/EBPβ and GR (*61*).

Another C/EBP family member, C/EBPδ, was shown to be induced by dexamethasone in 3T3-L1 preadipocytes (*12*) and adipocytes (*67*). In our preliminary experiments, dexamethasone also caused C/EBPδ mRNA induction in primary hepatocytes (Matsuno, F. and Iwase, K., unpublished results). Since a heterodimer can be formed between C/EBPβ and C/EBPδ (*12, 17, 68*), this heterodimer species may accumulate along with each homodimer in response to glucocorticoids, and may participate in the secondary response. Recently, another transcription factor named peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear receptor superfamily, was shown to be induced by glucocorticoids in primary-cultured rat hepatocytes (*36*). The possible cooperation or differential roles of C/EBPs and this factor in the secondary glucocorticoid response in the liver deserve further attention.

As mentioned above, C/EBPβ is likely to be regulated through phosphorylation in response to glucagon. Since the cAMP pathway is widely accepted as the major signaling pathway of glucagon, previous reports on activation of C/EBPβ by the cAMP pathway in PC12 cells (*46*) and phosphorylation of C/EBPβ by protein kinase A *in vitro* (*50*) are noteworthy. In addition, C/EBP family members and CREB/ATF family members seem to modulate each other's functions by forming a heterodimer (*69-71*), by sharing the target DNA sequence (*25, 45, 63, 72-74*), and by cooperating with each other by binding to different sites (*45, 75*).

Alongside these C/EBPβ-mediated pathways, potentially a number of other pathways, some of which were described above, seem to participate in transcriptional regulation in response to glucocorticoids and glucagon, altogether determining the final transcriptional levels of target genes.

In general, liver-specific traits such as gluconeogenesis (*1, 3*) and urea biosynthesis (*2, 4*) are stimulated cooperatively by glucocorticoids and glucagon. As described above, C/EBP family members interact with the regulatory regions of the genes for phosphoenolpyruvate carboxykinase (*25, 45, 63*), carbamylphosphate synthetase (*55*), and arginase (*56, 57*). The cooperative induction of these genes by glucocorticoids and glucagon might be accounted for, at least in part, by convergence of the effects of these hormones on activation of the C/EBPβ gene. It is tempting to speculate that C/EBPβ is generally involved in the regulation of gluconeogenesis and urea biosynthesis.

We thank D. Muller for the facilitation in preparing the manuscript, M. Ohara for the helpful comments, and our colleagues for the suggestions and discussions.

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