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Growth-Dependent Inhibition of CCAAT Enhancer-Binding Protein (C/EBPα) Gene Expression during Hepatocyte Proliferation in the Regenerating Liver and in Culture

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As an approach to understanding physiological mechanisms that control the proliferation of highly differentiated cells, we are addressing whether certain hepatic transcription factors participate in mechanisms that control the growth of hepatocytes. We have focused on CCAAT enhancer-binding protein (C/EBPα), a transcription factor which is highly abundant in normal liver and is considered to regulate expression of many genes, including some involved in energy metabolism (S. L. McKnight, M. D. Lane, and S. Gluecksohn-Walsh. Genes Dev. 3:2021–2024, 1989). Using Northern (RNA) blot analysis, we have examined the expression of C/EBPα mRNA during liver regeneration and in primary cultures of hepatocytes. C/EBPα mRNA levels decrease 60 to 80% within 1 to 3 h after partial hepatectomy as the cells move from G_0 to G_1 and decrease further when cells progress into S phase. Run-on transcription analysis is in agreement with the Northern blot data, thus suggesting that C/EBPα is transcriptionally regulated in regenerating liver. C/EBPα mRNA expression also decreases dramatically during the growth of freshly isolated normal hepatocytes cultured under conventional conditions (on dried rat tail collagen; stimulated to proliferate by epidermal growth factor [EGF] and insulin). Cultures of hepatocytes on rat tail collagen in the presence or absence of EGF clearly show that within 3 h, EGF depresses C/EBPα mRNA expression and that this effect is substantially greater by 4 h. Inhibition of protein synthesis in the liver by cycloheximide or in cultured hepatocytes by puromycin or cycloheximide effectively blocks the down-regulation of C/EBPα gene expression, apparently by stabilizing the normal rapid turnover of the C/EBPα mRNA (half-life of <2 h). This drop in C/EBPα gene expression in response to activation of hepatocyte growth is consistent with the proposal that C/EBPα has an antiproliferative role to play in highly differentiated cells (R. M. Umek, A. D. Friedman, and S. L. McKnight, Science 251: 288–292, 1991).

To investigate physiological mechanisms that may regulate proliferation of normal, highly differentiated cells, we are studying hepatocytes, which although they are quiescent in the liver, can proliferate vigorously in response to partial hepatectomy while retaining a nearly full complement of hepatocytic functions (20). Recent studies by Umek et al. (42) may provide some insight into the fundamental processes of growth and differentiation. These investigators propose that a particular transcription factor, CCAAT enhancer-binding protein (C/EBPα), has a major role to play in regulating the balance between these two processes in higher animals.

C/EBPα was first isolated from rat liver nuclear extracts as a sequence-specific, heat-stable DNA-binding protein that recognizes both the CAAT box and the enhancer core of several viral transcriptional control elements (25, 28). This protein has now been shown to belong to a class of transcription factors that contain a leucine zipper and a flanking basic DNA-binding domain (28). Additional members of the C/EBP family have been cloned and sequenced (1, 9, 11, 17, 35, 43); at least three members, C/EBPα, C/EBPβ, and C/EBPδ, show a strong amino acid sequence similarity within the basic region and leucine zipper domains (9, 43). All three proteins can form heterodimers with one another (9, 43), suggesting that they make up a functional family similar to those already described for other transcription factors.

C/EBPα is expressed predominantly in tissues carrying out gluconeogenesis and lipogenesis, most notably liver and fat. In these tissues, it appears to regulate expression of a spectrum of genes, some of which are involved in controlling energy metabolism (31). In the nongrowing and actively metabolizing normal adult liver, C/EBPα expression is high and restricted to the hepatocytes (4). Conversely, in rapidly growing hepatoma cells, C/EBPα expression is extremely low (19). These observations suggest that C/EBPα expression is inversely related to the proliferative state of the cell and are consistent with a role for C/EBPα as a factor that can function to maintain the quiescent state (42). In support of this hypothesis, our studies demonstrate that C/EBPα gene expression is rapidly and extensively down-regulated during proliferation of hepatocytes in the regenerating liver and in culture. This response can be potentiated in culture by epidermal growth factor (EGF) and is regulated at both a transcriptional and posttranscriptional level.

MATERIALS AND METHODS

Partial hepatectomy. Approximately 70% of the liver was surgically removed as described previously (23) and allowed to regenerate for various periods of time. Control animals were either normal intact rats or rats subjected to a sham operation, which consisted of a midline incision and a brief prodding of the intestines with a cotton swab to induce

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additional stress prior to wound closure, direct contact with the liver being carefully avoided. Where indicated, rats were injected with cycloheximide dissolved in sterile saline (50 mg/200 g of body weight) 15 min prior to surgery.

Hepatocyte isolation and culturing. Rat hepatocytes were isolated by a modified collagenase perfusion system (3, 39) and cultured under conventional conditions on dried rat tail collagen in Williams E medium supplemented with linoleic acid (50-μg/ml)-albumin (1-mg/ml) complex and 5×10^{-9} M dexamethasone, 1.3×10^{-7} M insulin, 20 mM pyruvate, and usually EGF (10 ng/ml). Cycloheximide (10 μg/ml), actinomycin D (5 μg/ml), and puromycin (10 μg/ml) were added where indicated.

RNA analysis. Total RNA was isolated from livers or hepatocytes by a modification of a procedure of Chomczynski and Sacchi (12). The tissue or cells were homogenized directly in extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% Sarkosyl, 0.1 M beta-mercaptoethanol) and subjected to exhaustive phenol-chloroform extraction, isopropanol precipitation, and removal of DNA by suspension in 4 M LiCl. Twenty-five micrograms of each RNA sample was analyzed by Northern (RNA) blot hybridization as described previously (5).

Isolation of hepatocyte nuclei from whole liver and run-on transcription assays. (Note that the isolation procedure was carried out in a cold room [4°C].) About 2 to 3 g of liver were homogenized in 18 ml of homogenizing buffer (0.34 M sucrose, 1 mM dithiothreitol, 10 mM magnesium acetate) and then centrifuged at 1,000 × *g* for 10 min. The crude nuclear pellet was fractionated further by resuspension in a solution of 30 ml of 2 M sucrose, 1 mM dithiothreitol, and 10 mM magnesium acetate followed by centrifugation at 21,000 rpm for 40 min in an SW28 rotor. This pellet was washed twice by suspension in nuclear storage buffer (40% glycerol, 50 mM Tris [pH 7.5], 1 mM dithiothreitol, 10 mM magnesium acetate) followed by low-speed centrifugation (1,000 × *g* for 10 min). The final nuclear pellet was resuspended in 250 μl of nuclear storage buffer for storage at −80°C until ready for use. For run-on assays, nuclear incorporation of [^{32}P]UTP in vitro was carried out essentially as described by Greenberg and Ziff (21) and modified by Bellas et al. (2).

cDNAs used were as follows: mouse C/EBPα (45), rat C/EBPα (28), albumin (gift from Douglas Cooper and Harvey Lodish), α1-antitrypsin (a gift from Harvey Lodish), histone 3.2 (16), c-*myc* (15), c-*jun* and *junB* (37), fibronectin (38), actin (5), α-tubulin (5), β-tubulin (6), and *egr-1* (41).

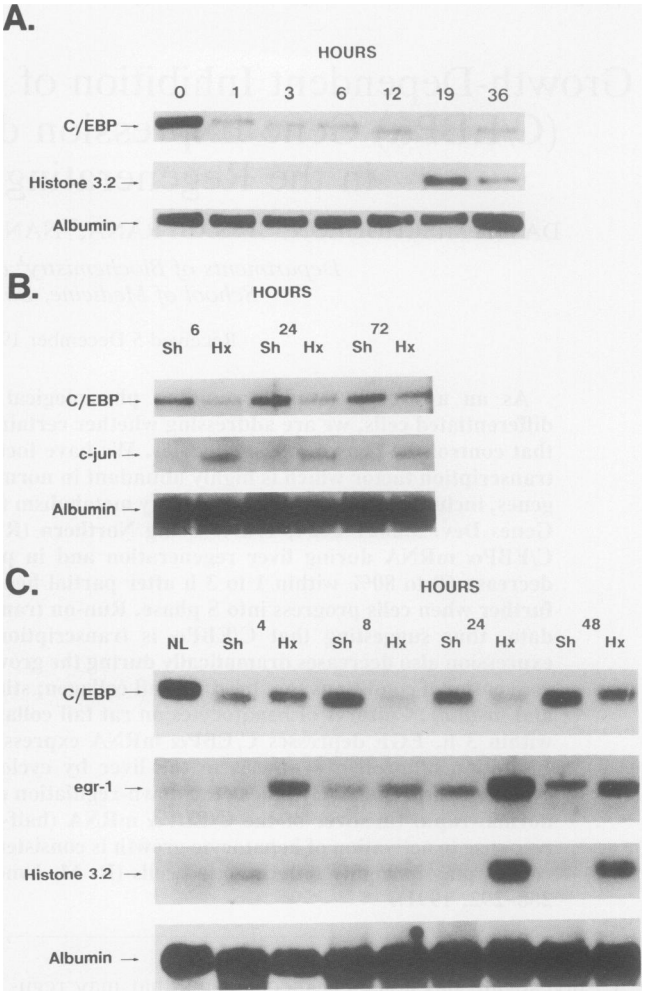

FIG. 1. Abundance of C/EBPα mRNA during liver regeneration. (A) Northern blot analysis of total RNA (25 μg) isolated from livers at the indicated times following a two-thirds partial hepatectomy. The mRNAs shown correspond to cDNAs used: rat C/EBPα, albumin, and histone 3.2. (B) Northern blot of total RNA isolated from regenerating livers (Hx) at the indicated times and from the corresponding sham controls (Sh). The blot was probed with C/EBPα, c-*jun*, and albumin cDNAs. (C) Northern blot of total RNA isolated from regenerating livers (Hx) at the indicated times and from corresponding sham controls (Sh). The blot was probed with C/EBPα, *egr-1*, histone 3.2, and albumin cDNAs.

RESULTS

Expression of C/EBPα mRNA during liver regeneration. The relative abundance of C/EBPα mRNA during liver regeneration was determined by Northern blot hybridization of total RNA from livers at various times following a partial hepatectomy. An abrupt drop in C/EBPα mRNA levels was observed within the first 3 h of regeneration (Fig. 1A and 2A). This corresponds to the time when the hepatocytes move from G_0 into G_1 phase of the cell cycle, as demonstrated by the activation of the immediate-early growth-associated genes, c-*jun* (Fig. 1B), and *egr-1* (Fig. 1C). The low level of C/EBPα expression remained quite constant until 19 h posthepatectomy, when there was a second drop corresponding to passage of cells into S phase, as indicated by induction of histone 3.2 mRNA (16).

Partial hepatectomy is known to induce a minor acute-phase response, presumably resulting from surgical stress (18). We therefore subjected a series of control rats to sham operations to assess what effect stress may have on C/EBPα mRNA expression. The data in Fig. 1C show a moderate decrease in C/EBPα mRNA levels in these control rats during the initial 1 to 4 h following surgery (Fig. 1C, compare lane 4 Sh with lane 4 Hx). This effect, however, appears to be a transient response, since C/EBPα mRNA levels in the sham-operated controls recover to near-normal amounts within 8 h postsurgery, whereas in the regenerating liver, C/EBPα mRNA abundance continues to fall abruptly to the low levels (10% of normal liver) expressed at the time of DNA synthesis (as indicated by an increase in histone 3.2 mRNA). After the initial transient decrease in C/EBPα mRNA in the sham-operated rats, levels returned to approximately 60% of the values in intact normal rats (Fig. 1C,

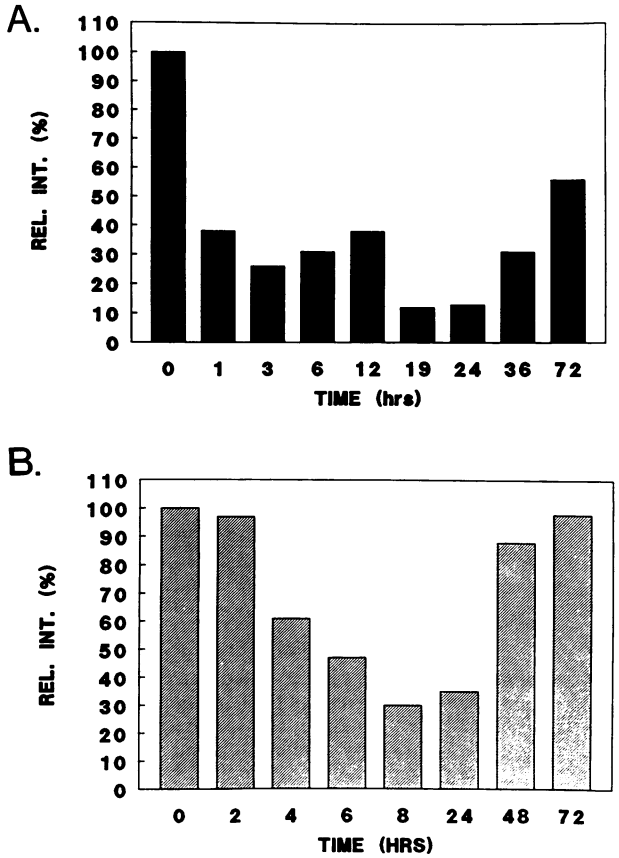

FIG. 2. Quantitation of Northern blot data from Fig. 1. (A) The intensity of the C/EBPα autoradiographic signal shown in Fig. 1A was quantified by laser densitometry and presented as a percentage of the control liver value, set at 100%. (B) The intensity of the C/EBPα autoradiographic signal in the regenerating liver relative to the intensity of the corresponding sham control at a given time point was quantified by laser densitometry.

compare lane 8 Sh with lane NL) and remained at this level throughout the entire postoperative period. On the other hand, for the hepatectomized animals, there was a gradual increase in C/EBPα mRNA after S phase (Fig. 1A, lane 36; Fig. 1C, lane 48 Hx; Fig. 1B, lane 72 Hx), returning to near-normal values by 72 h (Fig. 2B), a time when hepatic regeneration is beginning to shut down.

These observations may be more easily seen in the densitometer readings in Fig. 2. Figure 2A shows the pattern of C/EBPα mRNA expression in the regenerating liver relative to that of the normal untreated rats, with values normalized to the albumin signal at each time point in order to correct for unequal loading of the gel (Note that although some reports [33] suggest that albumin mRNA appears to increase during regeneration, we and others have found that it remains stable throughout the hepatectomy time course, and as such, it is a useful control). In Fig. 2B, the intensity of the regenerating liver C/EBPα signals obtained from the Northern blots is expressed relative to the signal from the sham-operated controls at the corresponding time point. When the data are viewed in this way, the extent of C/EBPα down-regulation is somewhat dampened. The extent of the decrease throughout the entire posthepatectomy period is less, because C/EBPα mRNA levels in the sham-operated animals remain at 50 to 60% the values expressed in normal liver. It is important to note that C/EBPα mRNA levels recover to the sham-operated control levels after the growth burst (72 h) rather than to the higher value found in normal liver.

Figure 1B and C also show an increase in the expression of the two immediate-early genes, c-*jun* and *egr-1* in response to partial hepatectomy. In the case of *egr-1*, the response to partial hepatectomy is biphasic, with an initial peak of expression occurring at 4 h followed by a more intense peak at 24 h postsurgery. The data also show a gradual increase in *egr-1* mRNA throughout the entire postsurgery period in the sham-operated control rats.

Transcriptional activity of the C/EBPα gene during liver regeneration. Many hepatic genes, both liver specific and growth associated, are regulated at a transcriptional level. We therefore performed a series of nuclear run-on assays to assess whether C/EBPα expression was similarly regulated. Figure 3 shows that transcription of the rat liver C/EBPα gene decreased after the hepatectomy, declined by 2-fold or more within 1 h and by 5- to 10-fold by 2 h, and remained at a low level of expression through S phase (Fig. 3A, lane 25 [25 h posthepatectomy]). Parallel to abundance of C/EBPα mRNA, transcription of C/EBPα gene increases as S phase subsides, reaching control levels by 72 h (note that the mC/EBP signal is stronger than rC/EBP signal because the mouse construct contains a larger fragment of the C/EBPα cDNA, and thus hybridizes with a greater amount of the radiolabelled RNA transcripts.) The increased transcription of actin (at 15 min), c-*myc* (at 60 min), and fibronectin (at 120 min) genes probably corresponds to the activation of a program of immediate-early genes accompanying the G_0-to-G_1 transition (10, 29, 33); the increased transcription of histone 3.2 gene, as noted earlier, is indicative of entry of hepatocytes into S phase at 24 h (8). α_1-antitrypsin gene transcription remained essentially constant throughout the time course, whereas albumin transcription decreased slightly (30%) possibly resulting from stress and/or diurnal fluctuations in hormones that alter some programs of hepatic gene expression (44). There is also a slight increase in the transcription of tubulin genes that may signify a growth-related change in the expression of components of the cytoskeleton during liver regeneration (8).

Figure 3B illustrates the pattern of C/EBPα transcription during a 72-h period posthepatectomy and includes assays performed on sham-hepatectomized animals at 1 and 6 h; in each case, there is no effect of surgery on C/EBPα transcription. The data show, in agreement with the Northern blot data from Figure 1, that the decrease in C/EBPα transcription correlates closely with the passage of hepatocytes through the cell cycle in the regenerating liver.

Effect of EGF on the Expression of C/EBPα mRNA in short-term cultures of hepatocytes. The kinetics of inhibition of C/EBPα gene expression during liver regeneration shown in Fig. 1 and 2 suggested that this process may be regulated by effectors that control the activation of quiescent hepatocytes into the cell cycle. EGF has been shown to be a potent hepatic mitogen both in animals and in culture (7, 36). We therefore assessed whether the expression of C/EBPα in cultured hepatocytes was influenced by EGF. Figure 4A shows a Northern blot analysis of total RNA isolated from hepatocytes cultured on rat tail collagen in the presence or absence of EGF. It is important to note that the level of C/EBPα mRNA in freshly isolated hepatocytes prior to plating is significantly lower than those levels measured in whole liver (Fig. 4, compare lane L with lane 0). Studies by

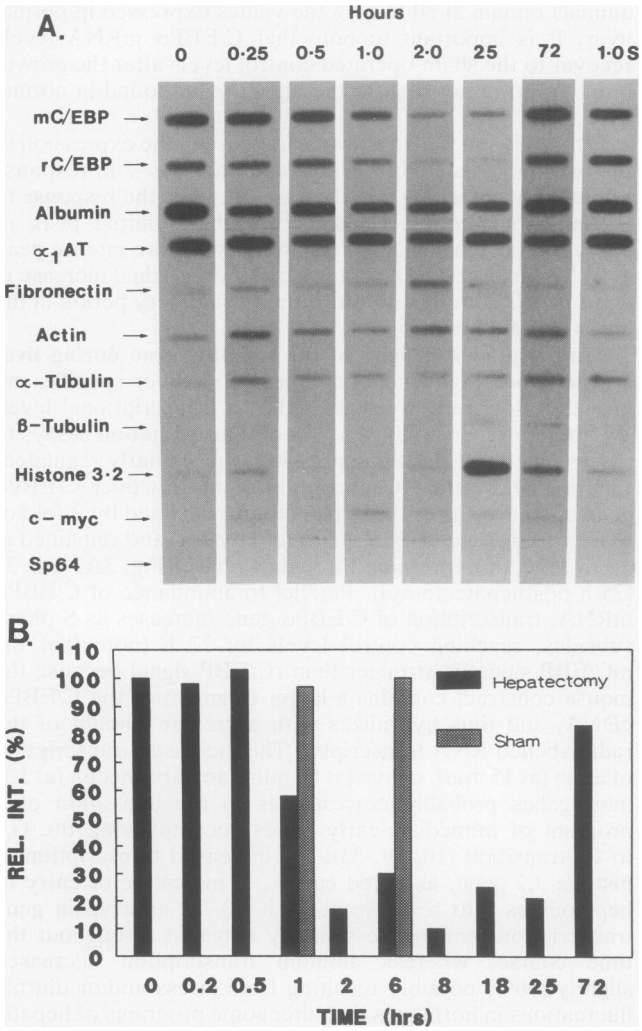

FIG. 3. Transcriptional activity of the C/EBPα gene relative to other abundant liver genes during liver regeneration. (A) Nuclear run-on transcription assays were performed on equal numbers of nuclei (10^7) isolated from the liver at the indicated times following a partial hepatectomy. Since the incorporation of counts varied with each nuclear preparation, hybridization was carried out with equal numbers of counts per each set. In vitro-extended ^{32}P-labelled RNA transcripts were hybridized to 10-μg samples of the indicated cDNAs immobilized on nitrocellulose. Hybridization was carried out at 65°C for 48 h as outlined in Materials and Methods. Lane 1.0S contains nuclei from a sham-operated rat 1 h after the operation. The genes assayed correspond to the various cDNA probes used, including mC/EBPα (a mouse cDNA probe), rC/EBPα (a rat cDNA probe), α_1AT, (α_1-antitrypsin), and Sp64 (control plasmid). (B) The intensity of the C/EBPα autoradiographic signal shown in panel A was quantified by laser densitometry and is presented as a percentage of the control normal liver value, set at 100%.

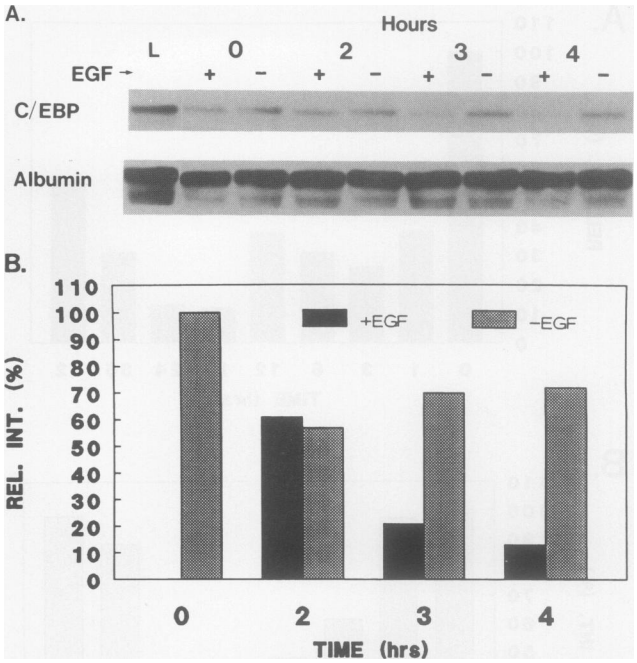

FIG. 4. Effect of EGF on the expression of C/EBPα mRNA in hepatocytes cultured under conventional conditions. (A) Northern blot analysis of total RNA (25 μg per lane) isolated from hepatocytes cultured under conventional conditions on dried rat tail collagen in the absence (−) or presence (+) of 10 ng of EGF per ml. The same blot was first hybridized with rC/EBPα cDNA, stripped, and rehybridized with albumin cDNA. (B) The intensity of the C/EBPα autoradiographic signal was quantified by laser densitometry and presented as a percentage of the signal obtained for the zero time point (cells obtained at time of plating).

ourselves (unpublished data) and others (45) have shown that the collagenase perfusion procedure employed to release hepatocytes is itself sufficient to initiate the G_0-to-G_1 transition even before the cells are plated, as evidenced by the activation of c-*fos*, c-*myc*, c-*jun*, and *junB* and by reduced transcription of C/EBPα. These activated cells do not appear to progress through G_1 until plated onto the appropriate substratum in the presence of EGF and insulin. EGF causes a further significant decrease of C/EBPα mRNA expression by 3 h; the decrease is most pronounced by 4 h.

Down-regulation of C/EBPα mRNA during liver regeneration and in cultured hepatocytes is dependent on protein synthesis. Many early growth responses can be activated in the absence of protein synthesis, as is well-known for immediate-early genes. We questioned whether the growth-related down-regulation of C/EBPα gene expression was similarly independent of hepatic translation. Injection of cycloheximide, an effective inhibitor of protein synthesis, into the portal vein 15 min prior to partial hepatectomy prevented the usual posthepatectomy decline of C/EBPα mRNA levels. Inhibition of hepatic protein synthesis with cycloheximide prevented the decline in C/EBPα mRNA levels, resulting in values comparable to those for normal liver throughout the 4.5-h period postsurgery (Fig. 5). As a control for adequacy of drug dosage, we analyzed the expression of three immediate-early genes, c-*myc*, c-*jun*, and *junB*, known to be superinduced in liver by cycloheximide (30, 33). As shown in Fig. 5, the drug dosage in this experiment was highly effective in superinducing all three of these genes, while C/EBPα mRNA was maintained at control liver levels. In EGF-stimulated cultures, the results of cycloheximide treatment are the same as within the animal. Figure 6A shows an extensive decrease in C/EBPα mRNAs in hepatocytes cultured during an 8-h period under conventional growth conditions (Fig. 6A, lanes 2, 6, 10, and 14), whereas culture of cells in the presence of 10 μg of cyclo-

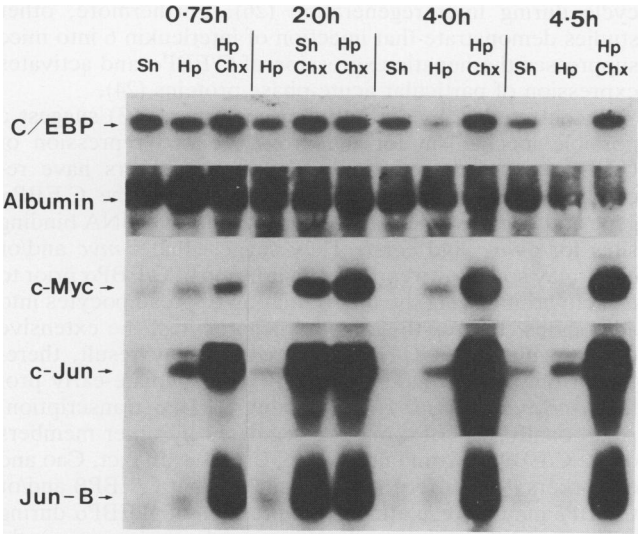

FIG. 5. Inhibition of hepatic protein synthesis with cycloheximide prevents the down-regulation of C/EBPα mRNA in the regenerating liver. Rats were hepatectomized and, where indicated, injected with cycloheximide (50 mg/200 g body weight) 15 min prior to surgery. RNA was isolated at the indicated times and subjected to Northern blot analysis as described in the legend to Fig. 1. Sh, sham; Hp, hepatectomy; Chx, cycloheximide.

heximide per ml (Fig. 6A, lanes 4, 8, 12, and 16) prevented the decrease in C/EBPα gene expression, resulting in relatively constant levels of C/EBPα mRNA during the entire 8 h. As seen in the animal, superinduction of c-*jun* mRNA indicates the adequacy of the drug dosage.

Expression of C/EBPα mRNA is regulated both at the levels of transcription and mRNA turnover in proliferating hepatocytes in culture. Earlier studies by Darnell and coworkers (45) demonstrated that the extensive drop in C/EBPα mRNA levels in freshly isolated hepatocytes cultured under conventional conditions resulted from a 10- to 20-fold decrease in transcription of the C/EBPα gene. It is conceivable, therefore, that cycloheximide may prevent the decrease in C/EBPα mRNA by inhibiting the synthesis of a nuclear factor that suppresses C/EBPα gene transcription. To assay for such an effect, we measured C/EBPα mRNAs in hepatocyte cultures containing cycloheximide and actinomycin D, an inhibitor of total RNA transcription. C/EBPα mRNA appears to be a very short-lived molecule (half-life of <2 h), since treatment of cells with actinomycin D alone for 2 h resulted in a level of C/EBPα mRNA at least 70 to 80% lower than that measured in untreated cells (Fig. 6A, compare lanes 2 and 3) and after 8 h of culture in actinomycin D, the mRNA was barely detectable (Fig. 6A, lane 15). Simultaneous inhibition of both transcription and protein synthesis resulted in sustained high levels of C/EBPα mRNA throughout an 8-h culture period (Fig. 6A, lane 17), suggesting that C/EBPα gene expression is regulated at a posttranscriptional and transcriptional level. In contrast, c-*jun* mRNA levels decreased substantially in cells treated with both drugs, indicating that c-*jun* is controlled primarily at the level of transcription.

As far as posttranscriptional mechanisms are concerned, cycloheximide may alter the rate of turnover of the C/EBPα mRNA by inhibiting the synthesis of a labile ribonuclease. Alternatively, since cycloheximide inhibits the elongation step of translation, resulting in the accumulation of ribosomes on mRNAs, its stabilizing effect may result from protection of C/EBPα mRNA from ribonucleases within a large polysomal structure. To determine which mechanism is involved, we blocked hepatocyte protein synthesis with puromycin, a drug which inhibits elongation by activating a premature release of ribosomes to generate unprotected nonpolysomal mRNPs. Figure 6B shows that treatment of hepatocytes with 10 μg of puromycin per ml prevented the drop in C/EBPα mRNA levels observed in untreated cells (compare lanes 2 and 6), thus having essentially the same effect as cycloheximide. In addition, this effect of puromycin was also due to a stabilization of C/EBPα mRNA, as indicated by abundant levels of C/EBPα mRNA in the actinomycin D- and puromycin-treated cells (Fig. 6B, lane 8). Therefore, C/EBPα mRNA turnover may be dependent

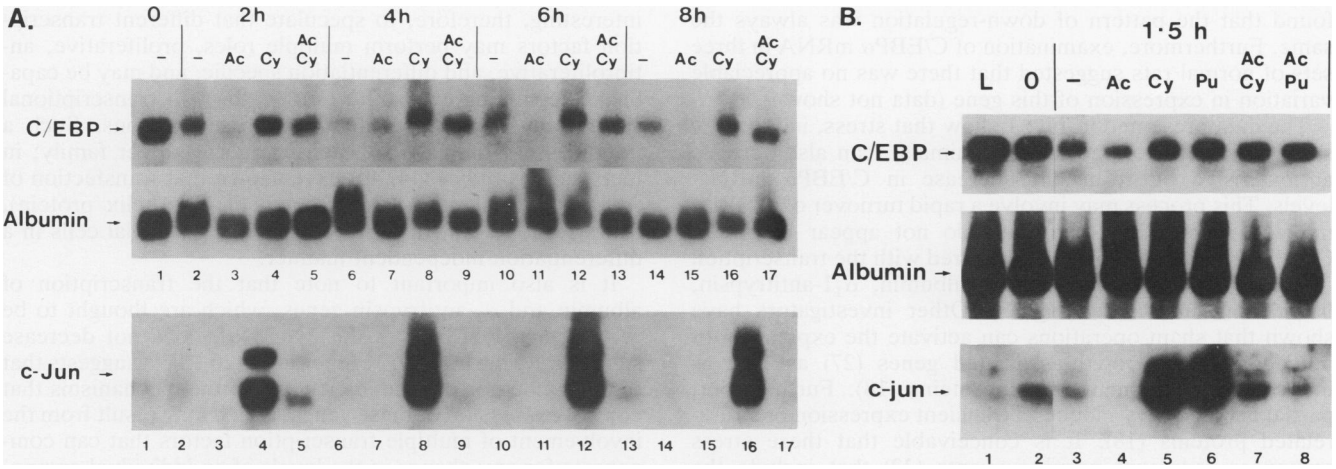

FIG. 6. Effect of inhibition of protein synthesis on the turnover of C/EBPα mRNA in cultured hepatocytes. Northern blot analysis of total RNA isolated from hepatocytes cultured under conventional conditions on rat tail collagen plus EGF and insulin in the presence or absence (–) of the following drugs: 5 μg of actinomycin (Ac) per ml, 10 μg of cycloheximide (Cy) per ml, and 10 μg of puromycin (Pu) per ml. Drugs were added either 30 (A) or 120 (B) min (zero time point in each case) after hepatocytes were plated onto the rat tail collagen substratum. Total RNA was isolated from the cells at the indicated times and subjected to Northern blot analysis as described in the legend to Fig. 4. Lane L, RNA from a normal rat liver; 0, RNA from freshly isolated hepatocytes prior to plating.

on the continuing synthesis of a labile ribonuclease that selectively degrades C/EBPα mRNA.

DISCUSSION

In regard to the mechanisms responsible for activating cells into the cell cycle, most studies have focused on the induction of a set of positive transcriptional activators such as the immediate-early gene products c-*fos*, c-*jun*, and c-*myc*. Very little attention has been given to the expression of genes that may need to be suppressed during growth activation. In these studies, we show that C/EBPα is rapidly down-regulated during the proliferation of hepatocytes in the regenerating liver (Fig. 1 and 2) and in culture (Fig. 4 and 6). Furthermore, expression appears to be controlled by the cell cycle, since C/EBPα gene transcription recovers in the liver soon after mitosis, a time when regeneration is beginning to slow down (Fig. 1 to 3). Additionally, we demonstrate that EGF, a potent hepatic mitogen, can potentiate the decrease in C/EBPα mRNA expression in cultured hepatocytes (Fig. 4), and this process appears to be dependent on prior protein synthesis (Fig. 5 and 6). Our data suggest that a labile protein participates in the selective degradation of C/EBPα mRNA in proliferating hepatocytes in culture, giving rise to an mRNA molecule with a very short half-life (<2 h). Whether the rapid turnover of C/EBPα mRNA is limited to proliferating cells is unsettled, because the decay of C/EBPα mRNA in the normal quiescent liver has not been determined. Additionally, our data show that the growth-related suppression of C/EBPα gene expression is regulated at the level of transcription. In fact, the cycloheximide-induced block of the down-regulation process may result from inhibition of the synthesis of labile proteins which suppress C/EBPα transcription during growth. The predominant stabilization of the C/EBPα mRNA by cycloheximide, however, prevents us from assessing, using Northern blots alone, the extent of such transcriptional control. Therefore, we are presently performing in vitro transcription run-on assays in nuclei isolated from cycloheximide-treated livers and hepatocytes in culture in order to address this question directly.

To ensure that the changes in C/EBPα expression seen here were due to growth, and not to variations in the animals (4), we repeated these experiments three to five times, and found that the pattern of down-regulation was always the same. Furthermore, examination of C/EBPα mRNA in three sets of normal rats suggested that there was no appreciable variation in expression of this gene (data not shown).

The data presented in Fig. 1 show that stress, induced by surgery (sham-operated control animals), can also activate an extensive but transient decrease in C/EBPα mRNA levels. This process may involve a rapid turnover of C/EBPα mRNA, since sham operations do not appear to inhibit C/EBPα transcription when compared with the transcription of other hepatic genes such as albumin, $\alpha_1$1-antitrypsin, fibronectin, and actin (Fig. 3). Other investigators have shown that sham operations can activate the expression of immediate-early growth-associated genes (27) as well as induce particular acute-phase proteins (18). Furthermore, partial hepatectomy induces a transient expression of stress-related proteins (18). It is conceivable that these stress responses represent priming events (32) that include the initial rapid decrease in C/EBP mRNA and that the sustained suppression of C/EBP gene transcription is associated with progression of hepatocytes through the cell cycle. In this regard, a recent study suggests that the cytokine interleukin 6 is involved in priming the hepatocytes to enter the cell cycle during liver regeneration (26). Furthermore, other studies demonstrate that injection of interleukin 6 into mice suppresses the hepatic expression of C/EBPα and activates expression of particular acute-phase proteins (24).

Recent studies by Christy and coworkers (13) suggest a possible mechanism for the growth-related repression of C/EBPα gene transcription. These investigators have recently characterized the promoter region of the C/EBPα gene and demonstrate the presence of putative DNA binding sites for c-*myc* and *egr-1*. They suggest that c-*myc* and/or *egr-1* may somehow repress transcription of C/EBPα prior to its activation during the differentiation of preadipocytes into adipocytes. In growth-activated hepatocytes, the extensive down-regulation of C/EBPα expression may result, therefore, from a rapid induction of these immediate-early proteins, c-Myc and Egr-1, that inhibit C/EBPα transcription. Additionally, C/EBPα may be regulated by other members of the C/EBP isoform family, e.g., C/EBPβ. In fact, Cao and coworkers (9) in a recent study suggest that C/EBPβ and/or C/EBPδ may be regulating the expression of C/EBPα during adipocyte differentiation. In this regard, we have recently identified at least three C/EBP binding sites within the C/EBPα promoter, and one of these sites is in close proximity to the c-*myc* and *egr-1* sites. Additionally, we have shown that C/EBPα can transactivate its own promoter in transient transfection assays (32a).

The data presented here is consistent with the hypothesis of Umek et al. (42) that C/EBPα maintains the highly differentiated state of specialized cells by regulating growth as well as the expression of tissue-specific genes. This hypothesis stemmed from a series of studies on the expression of conditional mutants of C/EBPα protein in proliferating preadipocytes. In these investigations, Umek et al. (42) demonstrated that the premature expression of C/EBPα in adipoblasts caused a direct cessation of mitotic growth independent of the differentiation process. C/EBPα may be one of many hepatic nuclear factors that function to maintain the quiescent state. In particular, it is likely that the other C/EBP isoforms may also be growth suppressors. Similarly, recent studies have also shown that another liver-specific transcription factor (DBP) that is related to the C/EBPs, is similarly down-regulated during liver regeneration induced by injection of carbon tetrachloride into rats (34). It is interesting, therefore, to speculate that different transcription factors may perform multiple roles, proliferative, antiproliferative, and differentiation specific, and may be capable of regulating each other, either through transcriptional control or by direct protein-protein interactions. Such a notion is not limited to the basic/leucine zipper family; in fact, recent studies (14, 40) have shown that transfection of the myogenic factor, MyoD (an helix-loop-helix protein), can arrest the growth of fibroblasts and epithelial cells in a differentiation-independent manner.

It is also important to note that the transcription of albumin and α_1-antitrypsin genes, which are thought to be regulated by C/EBPα in the liver (19), does not decrease significantly during liver regeneration (20). This suggests that there is a degree of redundancy within the mechanisms that control expression of these genes, which may result from the involvement of multiple transcription factors that can compensate for any change in the levels of an individual component. Indeed, the upstream sequences of the albumin gene do contain several DNA-binding domains to different liver transcription factors (22).

An aspect of C/EBPα that may be of particular relevance to liver regeneration is its dual role as a regulator of both

metabolism and growth. The widely held view that the liver regenerates in response to the metabolic demands of the body points to the potential importance of an in-depth analysis of the mechanisms through which the multiple functions of C/EBPα are implemented, possibly exemplifying a molecular mechanism through which metabolic demands are translatable into growth regulation.

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