

Liver-Enriched Transcription Factors in Liver Function and Development. Part II: the C/EBPs and D Site-Binding Protein in Cell Cycle Control, Carcinogenesis, Circadian Gene Regulation, Liver Regeneration, Apoptosis, and Liver-Specific Gene Regulation

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This article is dedicated to Amanda Maria Schrem.

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Abstract—In the first part of our review (see *Pharmacol Rev* 2002;54:129–158), we discussed the basic principles of gene transcription and the complex interactions within the network of hepatocyte nuclear factors, coactivators, ligands, and corepressors in targeted liver-specific gene expression. Now we summarize the role of basic region/leucine zipper protein family members and particularly the albumin D site-binding protein (DBP) and the CAAT/enhancer-binding proteins (C/EBPs) for their importance in liver-specific gene expression and their role in liver function and development. Specifically, regulatory networks and molecular interactions were examined in detail, and the experimental findings summarized in this review point to pivotal roles of DBP and C/EBPs

in cell cycle control, carcinogenesis, circadian gene regulation, liver regeneration, apoptosis, and liver-specific gene regulation. These regulatory proteins are therefore of great importance in liver physiology, liver disease, and liver development. Furthermore, interpretation of the vast data generated by novel genomic platform technologies requires a thorough understanding of regulatory networks and particularly the hierarchies that govern transcription and translation of proteins as well as intracellular protein modifications. Thus, this review aims to stimulate discussions on directions of future research and particularly the identification of molecular targets for pharmacological intervention of liver disease.

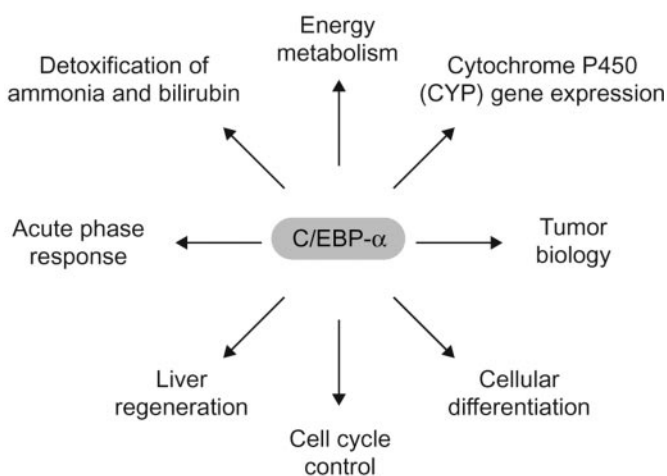
I. Introduction

New genomic platform technologies enable the study of complex genomes and proteomes for improved target identification and validation. This leads to high-density data sets on the order of millions of data points per day. Turning data into knowledge will be one of the biggest challenges of the 21st century. Here we describe concisely the role of liver-enriched transcription factors in regulatory gene networks and focus on liver development function and disease. This knowledge will prove to be indispensable for interpretation of high-density genomic data sets that are being produced in pharmaco- and toxicogenomics.

Indeed, numerous studies have established the pivotal role of liver-enriched transcription factors in organ development and cellular function, and there is conclusive evidence that transcription factors act in concert in liver-specific gene expression. During organ development and in progenitor cells, the timely expression of certain transcription factors is necessary for cellular differentiation, and there is overwhelming evidence for hierarchical and cooperative principles in a networked environment of transcription factors. The search for molecular switches that control stem cell imprinting and liver-specific functions has led to the discovery of many interactions among such different molecules as transcription factors, coactivators, corepressors, enzymes, DNA, and RNA. Many of these interactions either repress or activate liver-specific gene expression. In our first review we provided an overview of the basic principles of gene transcription and focused on the role of hepatic nuclear transcription factors in liver gene regulation (Schrem et al., 2002). Here we summarize the role of basic region leucine zipper (bZIP¹) protein family

¹Abbreviations: bZIP, basic region leucine zipper; DBP, D site-binding protein; C/EBP, CAAT/enhancer-binding proteins; AP-1, c-fos (transcription factor); CREB, cAMP responsive element-binding protein; BEF, bZIP-enhancing factor; ATF, activating transcription factor; CRE, cAMP response element; PEPCK, phosphoenolpyruvate carboxykinase; NF, nuclear factor; IL, interleukin; SAA, serum amyloid A; RDM, regulatory domain motif; ORF, open reading frame; bp, base pair; USF, upstream stimulating factor; T3, thyroid hormone; AUG, methionine; TE, transactivation element; CDP, CAAT displacement protein; HNF, hepatocyte nuclear factor; SL, stem loop; LAP, liver activating protein; LIP, liver inhibitory protein; LPS, lipopolysaccharide; TNF, tumor necrosis factor; RSK, ribosomal S kinase; APRF/STAT-3, acute phase response factor/signal transducers and activators of transcription; GADD, growth arrest and DNA damage inducible; A23187, 4-bromocalcymycin; MAP kinase, p38 mitogen-activated protein kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; CA-VI, murine carbonic anhydrase VI; CRU, cAMP response unit; CBP, CREB-binding protein; IRS, insulin response sequence; IGF1BP-1, insulin-like growth factor binding protein 1; GH, growth hormone; GSK-3, glycogen synthase kinase 3; P450, cytochrome P450; hnRNP, heterogeneous nuclear ribonucleoprotein; TGF, transforming growth factor; CDK, cyclin-dependent kinase; AD, activation domain; MnSOD, manganese superoxide dismutase; TNFRE, TNF response element; Con A, concanavalin A; HGF, hepatocyte growth factor; PH, partial hepatectomy; CLOCK, basic helix-loop-helix-PAS transcription fac-

A) Key Physiological Features of C/EBP- α



B) Key Physiological Features of C/EBP- β

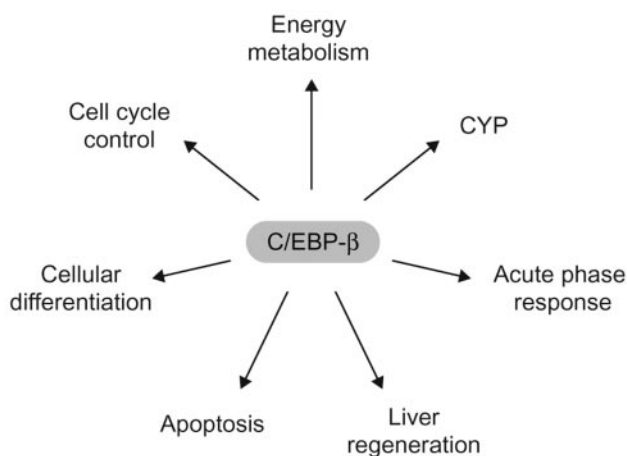


FIG. 1. The involvement of C/EBP- α and C/EBP- β in diverse physiological contexts highlights the important roles of these transcription factors in liver biology. A, the physiological contexts where C/EBP- α plays a major role; B, the same for C/EBP- β . Interestingly, C/EBP- α displays important functional features beyond the functions of a transcription factor that allows C/EBP- α to enable or inhibit proteasomal degradation of important regulatory molecules (e.g., C/EBP- β , CDK4, and p21) and to engage into inhibitory protein/protein interactions (e.g., disruption of E2F complexes). Details are discussed in the text.

members and focus on the albumin D site-binding protein (DBP) and the CAAT/enhancer-binding proteins (C/EBPs). Furthermore, we summarize our current knowledge on the roles of the transcription factors in important events like cell cycle control, carcinogenesis, liver regeneration, apoptosis, circadian gene regulation,

tor involved in the regulation of circadian gene expression; COX, cyclooxygenase; NS398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide; GST-P, glutathione *S*-transferase P; GPS1, GST-P silencer 1; PAR, proline and acidic amino acid residue-rich; TEF, thyroid embryonic factor; HLF, hepatic leukemia factor; PPAR, peroxisome proliferator activated receptor; nts, nucleotides; PP, peroxisome proliferator; DP, E2F-related transcription factor family.

and liver-specific gene regulation. As shown in Fig. 1, C/EBPs play a major role in diverse physiological processes.

Notably, several C/EBPs were discovered and characterized independently in different laboratories and given distinct names (Landschulz et al., 1988; Akira et al., 1990; Chang et al., 1990; Descombes et al., 1990; Poli et al., 1990; Roman et al., 1990; Ron and Habener, 1992; Takiguchi, 1998; Niu et al., 1999; Ramji and Foka, 2002). We followed the proposal by Cao et al. (1991), who suggested a systematic nomenclature in which members of the C/EBP subfamily are designated as C/EBP followed by a Greek letter indicating the chronological order of their discovery.

II. The CAAT/Enhancer-Binding Proteins

A. The C/EBP Subfamily of the Basic Region Leucine Zipper Family of Transcription Factors

A heat-stable DNA-binding protein found in rat liver nuclei was shown to be capable to bind selectively to the CCAAT motif of several viral promoters, as well as to the "core homology" sequence of several viral enhancers. Hitherto, this protein was termed CCAAT/enhancer-binding protein, or C/EBP (Landschulz et al., 1988). Later, it was recognized that several related proteins form a distinct subfamily of transcription factors called C/EBPs with several members (C/EBP- α , C/EBP- β , C/EBP- γ , C/EBP- δ , C/EBP- ϵ , and C/EBP- ζ). The C/EBP subfamily of transcription factors belongs to the larger family of bZIP transcription factors. Furthermore, important members of the bZIP family of transcription factors include c-jun, c-fos (AP-1), and the cAMP responsive element binding protein (CREB). The bZIP family of proteins is one of the largest and most conserved groups of eukaryotic transcription factors (Takiguchi, 1998; Niu et al., 1999).

The DNA-binding motif of transcription factors belonging to the bZIP family is bipartite, consisting of a dimerization interface termed "leucine zipper" and a DNA contact surface termed the "basic region." A common feature of the C/EBP subfamily members is that they consist of three structural components in a modular fashion that include a C-terminal leucine-zipper, a basic DNA-binding region, and an N-terminal transactivating region (Agre et al., 1989; Metallo and Schepartz, 1994; Niu et al., 1999; Ramji and Foka, 2002) (see also Fig. 2). The molecular chaperone bZIP-enhancing factor (BEF) was shown to increase DNA binding of transcription factors that contain a bZIP DNA-binding domain. BEF stimulates DNA binding by recognizing the unfolded leucine zipper and promoting the folding of bZIP monomers to dimers. Anti-sense experiments indicate that BEF is required for efficient transcriptional activation by bZIP proteins in vivo (Virbasius et al., 1999).

1. *C/EBP Homo- and Heterodimers.* Many transcription factors bind DNA to form dimeric (2:1) protein-

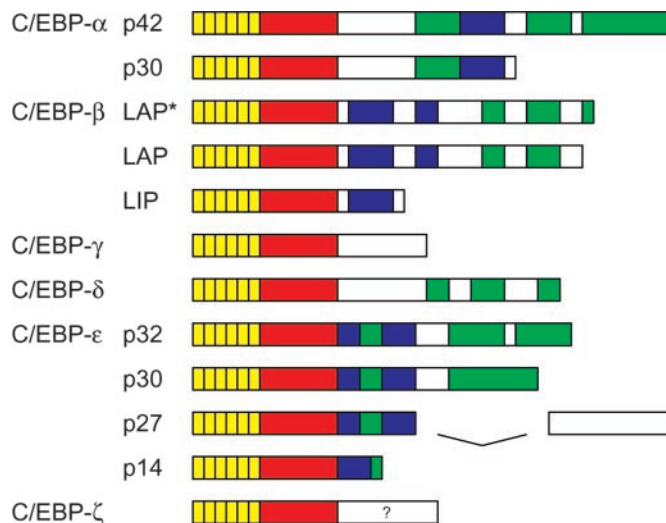


FIG. 2. A schematic model adapted with modifications from Ramji and Foka (2002) that highlights the modular structure of C/EBP subfamily members. The leucine zipper is depicted in yellow with black vertical lines indicating the leucine residues. The basic region is colored red. The position of the activation domains (ADs) and negative regulatory domains (RDs) are shown in green and blue, respectively. ? indicates that the N terminus of C/EBP- ζ contains an activation domain, although its exact position remains to be determined. The mechanisms for the formation of the different isoforms of C/EBP- α (p42, p30) and C/EBP- β (LAP*, p38; LAP, p35; LIP, p21) as well as their respective biological functions are described and discussed in the text. Although C/EBP- ϵ and C/EBP- ζ are members of the C/EBP subfamily, both do not belong to the exclusive group of liver-enriched transcription factors.

DNA complexes. Examples include bZIP proteins and basic region helix-loop-helix zipper proteins. These two families of transcription factors follow an assembly pathway in which two protein monomers bind DNA sequentially and form their dimerization interface while bound to DNA (Kohler et al., 1999). Dimerization of these transcription factors stabilizes the protein-DNA complexes and can lead either to homodimers with the same transcription factor or to heterodimers with other members of the same family of transcription factors (Horiuchi et al., 1997).

Noticeably, heterodimers between members of the C/EBP family and the CREB/ATF family of transcription factors (e.g., ATF-2, ATF-3, ATF-4, and C/ATF) were identified by several investigators (Vallejo et al., 1993; Shuman et al., 1997; Wolfgang et al., 1997; Fawcett et al., 1999). Both families belong to the larger group of bZIP proteins. The formation of C/EBP-ATF heterodimers causes altered DNA binding selectivity when compared with C/EBP and ATF homodimers. C/EBP-ATF heterodimers bind to a so-called C/EBP-ATF composite site in the promoter region of regulated genes. In particular, C/EBP α and ATF-2 homodimers bind cAMP response elements (CRE sites), but ATF-2-C/EBP- α heterodimers do not. Heterodimers bind an asymmetric sequence composed of one consensus half-site for each monomer and thus have a unique regulatory function (Shuman et al., 1997). C/ATF homodimers do not bind to typical C/EBP DNA sites. Instead, they bind to palin-

dromic CRE sites such as that of the somatostatin gene. C/ATF-C/EBP- β heterodimers bind to a subclass of asymmetric cAMP response elements exemplified by those in the phosphoenolpyruvate carboxykinase and proenkephalin genes (Vallejo et al., 1993).

2. C/EBPs and Cross-Talk with Other Transcription Factors. Transient transfection studies in HepG2 human hepatoma cells and in COS-1 monkey kidney cells indicated that interactions between C/ATF and C/EBP- β are the basis for a functional cross-talk between these two families of transcription factors that may be important for the integration of hormonal and developmental stimuli that determine the expression of subsets of genes in specific cellular phenotypes (Vallejo et al., 1993). By forming a complex with C/EBP- β , C/ATF directs binding of C/EBP- β away from C/EBP sites onto palindromic CREs. The relative rates of production and degradation of C/EBP- β and C/ATF might directly influence the regulation of many dependent genes. When the cellular concentrations of C/EBP- β are higher than the concentrations of C/ATF, this might favor C/EBP- β homodimer formation and, thus as a consequence, for example, angiotensinogen expression, whereas higher cellular C/ATF concentrations could favor C/ATF homodimer formation and, as a consequence, gene expression from genes bearing palindromic CREs, like that encoding somatostatin. Equal amounts of C/ATF and C/EBP- β might favor C/ATF-C/EBP- β heterodimer formation with the consequence of enhanced somatostatin, enkephalin, or phosphoenolpyruvate carboxykinase (PEPCK) expression (Vallejo et al., 1993). The formation of C/ATF-C/EBP- β heterodimers might also be influenced by post-translational modification of C/EBP- β by phosphorylation.

NF- κ B proteins and C/EBPs belong to distinct families of transcription factors that target unique DNA enhancer elements. The heterodimeric NF- κ B complex is composed of two subunits, a 50- and a 65-kDa protein. All members of the NF- κ B family, including the product of the proto-oncogene c-rel, are characterized by their highly homologous approximately 300-amino acid N-terminal region. This Rel homology domain mediates DNA binding, dimerization, and nuclear targeting of these proteins. A previously unexpected cross-coupling of members of the NF- κ B family with three members of the C/EBP family could be demonstrated in 1993. NF- κ B p65, p50, and Rel functionally synergize with C/EBP- α , C/EBP- β , and C/EBP- δ , respectively, via protein/protein interactions. Interestingly, this cross-coupling results in the inhibition of promoters with κ B enhancer motifs and in the synergistic stimulation of promoters with C/EBP binding sites (Stein and Baldwin, 1993; Stein et al., 1993). Protein/protein interactions of the bZIP region of C/EBP with the Rel homology domain of NF- κ B could be demonstrated for the regulation of interleukin 8 (IL-8) gene expression (Stein and Baldwin, 1993) as well as for the regulation of the major acute phase reactant serum amyloid A2 (SAA2) gene expression in the liver (Xia et

al., 1997) and in a protein complex in avian T cells that binds specifically to a consensus C/EBP site and contains both C/EBP and Rel family members (Diehl and Hannink, 1994).

3. Sumoylation May Modulate the Transactivation Activity of C/EBPs. A novel host cell post-translational modification system, termed sumoylation, has recently been characterized. Covalent modification of cellular proteins by the ubiquitin-like modifier SUMO regulates various cellular processes, such as nuclear transport, signal transduction, stress response, and cell cycle progression. But, in contrast to ubiquitylation, sumoylation does not tag proteins for degradation but seems to enhance their stability or modulate their subcellular compartmentalization (for recent reviews, see Muller et al., 2001; Wilson and Rangasamy, 2001). SUMO is highly conserved from yeast to humans. Whereas invertebrates have only a single SUMO gene, three members of the SUMO family have been described in vertebrates. Like ubiquitin, all SUMO forms are initially made as inactive precursors. The processing reaction is catalyzed by a group of cysteine proteases, termed ubiquitin-like protein-processing enzymes or SUMO-specific proteases. In contrast to ubiquitin, SUMO conjugation does not seem to lead to the formation of SUMO-SUMO chains on the substrate. Sumoylation is a dynamic, reversible process. Desumoylation is catalyzed by the ubiquitin-like protein-processing enzyme/SUMO-specific proteases (Muller et al., 2001; Wilson and Rangasamy, 2001).

In C/EBP- ϵ , an inhibitory domain termed regulatory domain I was characterized. It could be shown that functionally related domains are present in the liver-enriched transcription factors C/EBP- α , C/EBP- β , and C/EBP- δ . These domains contain an evolutionarily conserved five-amino acid motif [the regulatory domain motif (RDM)] that conforms to the consensus sequence [IVL]-K-x-E-P. Mutagenesis studies revealed that the residues at positions 1, 2, and 4 of the RDM are critical for inhibitory domain function. Importantly, the RDM is similar to the recognition sequence for attachment of the ubiquitin-like protein SUMO-1, and it could be shown that the conserved lysine residue of each C/EBP RDM served as an attachment site for SUMO-1. SUMO-1 attachment decreased the inhibitory effect of the C/EBP- ϵ regulatory domain, suggesting that sumoylation may play an important role in modulating C/EBP- ϵ activity, as well as that of the other C/EBP family members (Kim et al., 2002).

B. C/EBP- α (Originally Named C/EBP)

1. The Single-Exon C/EBP- α Gene Is Highly Conserved in Different Species. The C/EBP- α gene was found to be syntenic on human chromosome 19, on rat chromosome 1, and on mouse chromosome 7. These results provide further evidence for conservation of synteny (= genome collinearity) on these three chromosomes (Birkenmeier et al., 1989; Szpirer et al., 1992).

There is a strong conservation of C/EBP- α and its role in gene expression as demonstrated, for instance, by the regulation of the alcohol dehydrogenase gene in the adult body of *Drosophila melanogaster* and in the human and rat liver (Falb and Maniatis, 1992; Potter et al., 1994). Further, the human C/EBP- α gene is 2783 bp long and encodes a 356-amino acid protein, which is of the same length as the gene for rat C/EBP- α . Compared with rat C/EBP- α , there are two insertions of two amino acids and one deletion of four. The amino acid similarity between the two proteins is over 92% (Antonson and Xanthopoulos, 1995). The C/EBP- α mRNAs of chicken, rat, and *Xenopus* all contain a small 5' open reading frame (5'ORF), whose size (18 nucleotides) and distance (seven nucleotides) to the C/EBP- α cistron have been conserved in vertebrate evolution (Calkhoven et al., 1994).

2. Tissue-Specific and Species-Specific Autoregulation of the C/EBP- α Promoter. The human C/EBP- α gene was found to be expressed at highest levels in placenta. High expression was also found in liver, lung, skeletal muscle, pancreas, small intestine, colon, and in peripheral blood leukocytes, but the expression was undetectable or very low in brain, kidney, thymus, testis, and ovary (Antonson and Xanthopoulos, 1995). In vitro transcription experiments showed that the basis for the restricted cellular distribution of the mouse C/EBP- α mRNA is to be found in the transcriptional regulation of the gene (Xanthopoulos et al., 1989).

The sequence of the C/EBP- α promoter that includes the USF binding site is also capable of forming stable complexes with purified Myc+Max heterodimers, and mutation of this site drastically reduces transcription of C/EBP- α promoter luciferase constructs both in liver and nonliver cell lines (Legraverend et al., 1993). In addition, three additional protein-binding sites were identified, two of which display similarity to NF-1 and NF- κ B binding sites. The region located between nucleotides -197 and -178 forms several heat-stable complexes with liver nuclear proteins in vitro, which are recognized mainly by antibodies specific for C/EBP- α . Furthermore, transient expression of C/EBP- α and, to a lesser extent, C/EBP- β expression vectors result in transactivation of a cotransfected C/EBP- α promoter-luciferase reporter construct. These experiments support the notion that the gene coding for C/EBP- α is regulated by C/EBP- α , but other C/EBP-related proteins may also be involved in its regulation (Legraverend et al., 1993).

The human C/EBP- α gene promoter shares significant sequence homology with that of the mouse but interestingly has a different mechanism of autoregulation. Activation of the murine promoter by direct binding of C/EBP- α to a site within 200 bp of the transcriptional start was shown to elevate activity by approximately 3-fold (Legraverend et al., 1993; Timchenko et al., 1995). Unlike its murine counterpart, the human C/EBP- α

gene promoter does not contain a *cis* element that binds the C/EBP- α protein. Neither C/EBP- α nor C/EBP- β binds the human C/EBP- α promoter within 437 bp. However, cotransfection studies in human hepatoma-derived Hep3B2 cells show that C/EBP- α stimulates transcription of a reporter gene driven by 437 bp of the C/EBP- α promoter. The human C/EBP- α protein stimulates upstream stimulating factor (USF) to bind to a USF consensus element within the C/EBP- α promoter and activates it by 2- to 3-fold (Timchenko et al., 1995). Therefore, it was proposed that the human C/EBP- α gene uses the ubiquitously expressed DNA-binding protein factor USF to carry out autoregulation. Autoregulation of the human C/EBP- α promoter could be experimentally abolished by deletion of the USF binding site, CACGTG. Expression of human C/EBP- β after transfection did not stimulate USF binding. These studies suggest a mechanism whereby tissue-specific autoregulation can be achieved via a transacting factor that is expressed in all cell types. Thus, direct binding of the C/EBP- α protein to the promoter of the C/EBP- α gene is not required for autoregulation (Timchenko et al., 1995). Studies with the *Xenopus laevis* C/EBP- α promoter showed that, in contrast to the human promoter and in common with the murine gene, the xenopus C/EBP- α promoter was subject to direct autoregulation (Kockar et al., 2001).

3. Thyroid Hormone Positively Regulates C/EBP- α Expression. A genomic clone containing 1171 bp of the 5'-flanking region of the rat C/EBP- α gene was shown to be an active promoter in MB492 cells, which is an immortalized brown adipocyte cell line that expresses the endogenous C/EBP- α gene in a thyroid hormone (T3)-dependent manner. This genomic clone and the MB492 cell line were used for deletion, mutagenesis, and gel mobility shift experiments to further characterize the rat C/EBP- α promoter. Results from these experiments showed that the TRE-1 element (-602/-589) of the rat C/EBP- α promoter possesses a ER2-type response element that represents a functional T3 response element regulated by thyroid hormone and that thyroid hormone is a factor that positively regulates C/EBP- α gene expression in a direct fashion (Menendez-Hurtado et al., 2000). Furthermore, several peptide regions within the transactivation domain of C/EBP- α could be identified that enhance the ability of thyroid hormone to stimulate gene transcription (Jurado et al., 2002). Therefore, it can be assumed that C/EBP- α expression is enhanced by thyroid hormone 2-fold: directly via a T3 response element in the C/EBP- α promoter and indirectly by interaction with the transactivation domain of C/EBP- α leading to an enhanced C/EBP- α autoregulatory loop.

4. Possible Regulatory Function of Alternative C/EBP- α Translation Products. C/EBP- α mRNA is translated into two major proteins, p42-C/EBP- α and p30-C/EBP- α , that differ in their content of N-terminal amino acid sequences (Lin et al., 1993; Ossipow et al.,

1993). p30-C/EBP is an alternative translation product initiated at the third in-frame AUG (methionine) codon of the C/EBP- α message (Lin et al., 1993; Ossipow et al., 1993). It was demonstrated that the small 5'ORF is crucial to the leaky scanning mechanism of ribosomes causing a fraction of them to ignore the first C/EBP- α AUG codon and to start at internal AUGs (Calkhoven et al., 1994). The full-length p42-C/EBP- α acts as a transactivator in the liver, whereas the N-terminally truncated p30-C/EBP- α lacks transcription activation potential (Ossipow et al., 1993; Calkhoven et al., 1994). Unlike p42-C/EBP- α , which inhibits cell proliferation, p30-C/EBP- α is not antimitotic. Thus, the N-terminal 12-kDa segment of full-length C/EBP- α contains an amino acid sequence necessary for antimitotic activity. Furthermore, during differentiation of 3T3-L1 preadipocytes and during hepatocyte development, the cellular p42-C/EBP- α /p30-C/EBP- α ratio changes, raising the possibility of a regulatory role (Lin et al., 1993). The impact of C/EBP- α on cell cycle control as well as its role in development are discussed in the following paragraphs in greater detail.

5. *The Transactivation Domains of C/EBP- α .* The N-terminal portion of C/EBP- α contains three distinct domains. The first domain (amino acids 1 to 107 = TE I) appears to be a highly potent transactivator. The second domain (amino acids 107 to 170 = TE II) does not appear to exhibit either activation or repression activity. The third domain (amino acids 171 to 245 = TE III) is a relatively weaker transactivator with a striking proline-rich motif (Pei and Shih, 1991). The C/EBP- α transactivation domain thus contains three transactivation elements (TEs) that synergistically activate transcription in mammalian cells. Two of these elements, TE-I and -II, cooperatively mediate in vitro binding of C/EBP- α to TBP and TFIIB, two essential components of the RNA polymerase II basal transcriptional apparatus. The TBP and TFIIB binding elements of C/EBP- α coincide and require amino acid motifs conserved between the activating members of the C/EBP family. These same motifs are necessary for the transcription activation function of TE-I and -II in both yeast and mammalian cells. This indicates that this modularity is conserved in eukaryote evolution. It was suggested that domains of TBP and TFIIB that interact with activating surfaces are functionally similar and may be structurally related. This supports the idea that the same amino acid motifs in an activator carry out multiple functions during the initiation process (Nerlov and Ziff, 1995).

6. *RFC140 Acts as a Coactivator for C/EBP- α .* Western blotting experiments showed that the bZIP domain of C/EBP- α interacts with the DNA-binding region of RFC140, a large subunit of the replication factor C complex from rat liver nuclear extracts. Overexpression of RFC140 in mammalian cells increased the transactivation activity of C/EBP- α on both minimal and native promoters. Consistent with the enhanced transactivation,

a complex of C/EBP- α and RFC140 proteins with the cognate DNA element was detected in vitro. The specific interaction between C/EBP- α and RFC140 was detected in the terminal differentiation of 3T3-L1 preadipocytes to adipocytes. The synergistic transcription effect of these two proteins increased the promoter activity and protein expression of peroxisome proliferator-activated receptor- γ , which is a main regulator of adipocyte differentiation. These results demonstrate that the specific transcription factor C/EBP- α and the general DNA replication factor RFC140 interact functionally and physically (Hong et al., 2001). Noticeably, the levels of the general replication factor obviously modulate the functional activity of the specific transcription factor C/EBP- α as a coactivator.

7. *CAAT Displacement Protein as a Competitive Repressor for C/EBP- α -Mediated Transactivation.* The nuclear matrix protein CAAT displacement protein (CDP) was discovered as a competitive repressor for CCAAT binding factors in experiments on gene regulation of the sperm histone H2B-1 of the sea urchin *Psammechinus miliaris* by Barberis et al. (1987). In blastula and gastrula embryo extracts, CDP binds with high affinity to sequences overlapping the proximal CCAAT element of the sperm histone H2B-1 promoter, thus preventing the DNA interaction of the CCAAT-binding factor in the embryo where the sperm *H2B* gene is not expressed (Barberis et al., 1987).

Investigations on liver-specific gene regulation of the human cholesterol 7 α -hydroxylase *CYP7A1* gene revealed a transcriptional repressor within the first intron of *CYP7A1* in transient transfection experiments with HepG2 cells. In the first intron, three DNase I hypersensitivity sites were found with five binding sites for the nuclear matrix protein CDP (Antes et al., 2000). Further, a matrix attachment site was found throughout the entirety of intron 1 of the *CYP7A1* gene. Gel retardation experiments and cell transfection studies provided evidence for the repression mechanism by CDP. It was shown that repression of the human *CYP7A1* gene is mediated by the matrix attachment site-bound repressor CDP, involves displacement of two hepatic transcriptional activators, HNF-1 α and C/EBP- α , from their binding sites within intron 1 of the *CYP7A1* gene, and thus represses transactivation mediated by these two activators (Antes et al., 2000). The observation that CDP is abundant in undifferentiated cells and is down-regulated in differentiated epithelial cells (Ai et al., 1999) might indicate that the availability of CDP for its sites within intron 1 of the *CYP7A1* gene is high early in liver development and thus binding of the liver-specific transcription factors may be blocked (Antes et al., 2000). Later in development, when hepatocytes are further differentiated and when CDP expression may be down-regulated, binding by HNF-1 α and C/EBP- α may occur at a time when *CYP7A1* functions are needed metabolically.

8. *Translational Inhibition of C/EBP- α Expression by Calreticulin.* The process of “quality control” in the endoplasmic reticulum involves a variety of mechanisms that collectively ensure that only correctly folded, assembled, and modified proteins are transported along the secretory pathway. In contrast, non-native proteins are retained and eventually targeted for degradation. Recent work provides structural insights into the process of glycoprotein folding in the endoplasmic reticulum involving the lectin chaperone calreticulin (for review, see Cabral et al., 2001; Ellgaard and Helenius, 2001). Calreticulin is a calcium-binding protein of the endoplasmic reticulum that has been found to function as a nuclear export factor for a large family of nuclear receptors. Atypical nuclear export pathways may thus exist that regulate the compartmentalization and activity of a distinct set of transcription factors (DeFranco, 2001).

It could be demonstrated that calreticulin interacts with GCN repeats within C/EBP- α and C/EBP- β mRNAs. GCN repeats within these mRNAs form stable SL structures. The interaction of calreticulin with SL structures of C/EBP- α and C/EBP- β mRNAs leads to the inhibition of translation of C/EBP proteins in vitro and in vivo. Deletions or mutations abolishing the formation of SL structures within C/EBP- α and C/EBP- β mRNAs lead to a failure of calreticulin to inhibit translation of C/EBP proteins. Calreticulin-dependent inhibition of C/EBP- α is sufficient to block the growth-inhibitory activity of C/EBP- α . This finding further defines the molecular mechanism for post-transcriptional regulation of the C/EBP- α and C/EBP- β proteins (Timchenko et al., 2002).

C. C/EBP- β (Formerly Also Named Liver-Activating Protein, Interleukin 6DBP, CRP2, or Nuclear Factor Interleukin 6)

C/EBP- β is a 32-kDa protein that shares extensive sequence homology (71%) in its DNA-binding and leucine zipper domains with C/EBP- α . C/EBP- β and C/EBP- α can interact in vitro to form heterodimers that bind to DNA with the same specificity as the respective homodimers (Descombes et al., 1990; Poli et al., 1990).

1. *C/EBP- β Isoforms Are the Result of Alternative Translation Initiation.* Both C/EBP- α and C/EBP- β are intronless genes that can produce several N-terminally truncated isoforms through the process of alternative translation initiation at downstream AUG codons (Ossipow et al., 1993; Welm et al., 1999). C/EBP- β has been reported to produce four isoforms: full-length 38-kDa C/EBP- β , 35-kDa LAP (liver-enriched transcriptional activator protein), 21-kDa LIP (liver-enriched transcriptional inhibitory protein), and a 14-kDa isoform (Ossipow et al., 1993; Welm et al., 1999). The production of multiple proteins from a single strand of mRNA is not only shared between different C/EBP family members but also appears to be a conserved mechanism in verte-

brate evolution (Ossipow et al., 1993; Calkhoven et al., 1994; Welm et al., 1999).

The C/EBP- β mRNA has four in-frame AUGs and an internal out-of-frame AUG associated with a small ORF. Initiation of translation at the in-frame AUGs forms 40-kDa (AUG-1), 35-kDa (AUG-2), 20-kDa (AUG-3), and 8.5-kDa (AUG-4) isoforms (Xiong et al., 2001).

It is important to understand the regulation and the production of various C/EBP- β isoforms as they display different transcriptional capabilities. For instance, the truncated C/EBP- β isoform, LIP, is up-regulated in rat livers after partial hepatectomy via an alternative translation mechanism (Burgess-Beusse et al., 1999; Timchenko et al., 1999a; Welm et al., 1999). A number of mechanisms have been elucidated that lead to differential C/EBP- β isoform production, as detailed below.

2. *Liver-Specific Proteosomal Regulation of C/EBP- β Isoforms by C/EBP- α .* Using an in vitro translation system, it was found that LIP was produced by two mechanisms: alternative translation and a proteolytic cleavage of full-length C/EBP- β . In C/EBP- α knockout mice (C/EBP- $\alpha^{-/-}$), the regulation of C/EBP- β proteosomal degradation was impaired (Burgess-Beusse et al., 1999; Welm et al., 1999) (see also Fig. 5). Induction of C/EBP- α in cultured cells leads to induced cleavage of C/EBP- β to generate the LIP isoform. The cleavage activity in mouse liver extracts is specific to prenatal and newborn livers, is sensitive to chymostatin, and is completely abolished in C/EBP- $\alpha^{-/-}$ animals. The lack of cleavage activity in the livers of C/EBP- $\alpha^{-/-}$ mice correlates with the decreased levels of LIP in the livers of these animals (Burgess-Beusse et al., 1999; Welm et al., 1999).

3. *Translational Regulation of C/EBP- β Isoforms by CUGBP1.* CUG repeat binding protein, CUGBP1, interacts with the 5' region of C/EBP- β mRNA and regulates translation of C/EBP- β isoforms. Two binding sites for CUGBP1 are located side by side between the first and second AUG codons of C/EBP- β mRNA. One binding site is observed in- and out-of-frame short ORF that has been shown previously to regulate initiation of translation from different AUG codons of C/EBP- β mRNA. Analysis of cytoplasmic and polysomal proteins from rat liver after partial hepatectomy evidenced that CUGBP1 is associated with polysomes to translate low-molecular-weight isoforms of C/EBP- β . The binding activity of CUGBP1 to the 5' region of C/EBP- β mRNA shows increased association with these polysomal fractions after partial hepatectomy. Addition of CUGBP1 into a cell-free translation system leads to increased translation of low-molecular-weight isoforms of C/EBP- β . These data therefore demonstrate that CUGBP1 protein is an important component for the regulation of initiation from different AUG codons of C/EBP- β mRNA (Timchenko et al., 1999a).

4. *Translational Inhibition of C/EBP- β Expression by Calreticulin.* As described above for C/EBP- α , calreti-

culin is also able to interact with GCN repeats that form stable SL structures within C/EBP- β mRNAs, leading to an inhibition of C/EBP- β translation in vitro and in vivo (Timchenko et al., 2002).

5. Regulation of the C/EBP- β Promoter. Within the 5'-regulatory region of mouse C/EBP- β , three protein factor-binding motifs, named UF1 (-376 to -352), UF2 (-254 to -223), and UF3 (-220 to -190), as well as two Sp1 motifs (-309 to -277 and -264 to -241) were identified by DNase I footprinting assays using nuclear extracts from lipopolysaccharide (LPS)-stimulated or unstimulated livers. Biochemical analysis confirmed that C/EBP- β binds to the UF1 and UF2 sites, implying autoregulation of C/EBP- β during the acute phase response (Chang et al., 1995).

Deletion analysis of the 5'-flanking region of rat C/EBP- β , located upstream of the start site of transcription in the C/EBP- β gene, demonstrated that a small region in close proximity to the TATA box (bp -121 to -71) is important in maintaining high levels of transcription of luciferase reporter gene constructs. Electromobility shift experiments identified two sites that are important for specific complex formation within this region. Further analysis by cross-linking, super shift, and competition experiments with liver cell nuclear extracts, hepatoma cell nuclear extracts, or recombinant CREB protein demonstrated CREB binding to both sites in the C/EBP- β promoter with an affinity similar to that with the CREB consensus sequence. Moreover, transfection experiments with promoter constructs where the CREB sites were mutated showed that these sites are important to maintain both basal promoter activity and C/EBP- β inducibility through CREB. Western blot analysis of rat liver cell nuclear extracts and runoff transcription assays of rat liver cell nuclei after two-thirds hepatectomy showed a functional link between the induction of CREB phosphorylation and C/EBP- β mRNA transcription during liver regeneration (Niehof et al., 1997). As several pathways control CREB phosphorylation, these results provide evidence for the transcriptional regulation of C/EBP- β via CREB under different physiological conditions.

6. Tissue-Specific Autoregulation of C/EBP- β Transcription. The two cAMP responsive elements within the rat C/EBP- β promoter between nucleotides -121 and -71 play an important role in C/EBP- β autoregulation as demonstrated by deletion analysis and luciferase reporter gene experiments. Gel shift experiments using oligonucleotides with overlapping point mutations aided in the identification of the GCAATGA-sequence (β -site) adjacent to and partially overlapping the first CRE-like site as the core motif for C/EBP- β binding. Further analysis of a mutated β -site in reporter gene experiments showed the functional relevance of this site for autoregulation. The composite C/EBP- β -CRE-element in the promoter enabled synergistic activation of transcription by C/EBP- β and the CREB pathway in a cell type-spe-

cific manner. In HepG2 hepatoma cells, NF- κ B increased autoregulation and could therefore mediate enhanced activation during inflammatory responses (Niehof et al., 2001a). These results demonstrated that the assembly of the three binding sites within the C/EBP- β promoter and thus the interaction between C/EBP- β and members of the CREB or NF- κ B family allows the specific control of C/EBP- β gene transcription in different physiologic contexts such as inflammation or the acute phase response.

7. Negative Regulatory Domains of C/EBP- β . It could be demonstrated that C/EBP- β contains a negative regulatory region composed of two elements, called RD1 and RD2. Deletions of RD2 relieve the inhibition of C/EBP- β activity in intestinal endocrine L cells, but do not affect C/EBP- β function in HepG2 hepatoma cells. These deletions also increase the DNA binding activity of C/EBP- β approximately 3-fold, suggesting that RD2-mediated repression of DNA binding activity is responsible for C/EBP- β inhibition in L cells. The adjacent RD1 element functions independently of RD2 and modulates the C/EBP- β activation domain, which was shown to be composed of three subdomains that are conserved within the C/EBP protein family. RD1 does not affect cell type specificity, but it inhibits the transactivation potential of GAL4-C/EBP- β hybrid proteins by 50-fold. These findings suggest that C/EBP- β assumes a tightly folded conformation in which the DNA binding and activation domains are masked by interactions with the regulatory domain, and that to function efficiently in HepG2 cells, the protein must undergo an activation step. It was proposed that relief of inhibition conferred by the regulatory domains also accounts for C/EBP- β activation in response to extracellular signals (Williams et al., 1995). It was suggested that phosphorylation plays a unique role to derepress rather than to enhance the transactivation domain as a novel mechanism to regulate gene expression by C/EBP- β (Kowenz-Leutz et al., 1994).

8. Post-translational Phosphorylation of C/EBP- β . It could be demonstrated that post-translational site-specific phosphorylation of C/EBP- β is an essential mechanism in the regulation of C/EBP- β -dependent gene regulation (Mahoney et al., 1992; Trautwein et al., 1993, 1994; Buck et al., 1999). Phosphorylation of C/EBP- β at Ser299 and Ser277 by protein kinase C or by M-kinase resulted in an attenuation of binding to a 32 P-labeled CCAAT oligodeoxynucleotide in vitro (Mahoney et al., 1992). Phosphorylation of C/EBP- β by cAMP-dependent protein kinase A or by protein kinase C at Ser240 within the DNA-binding domain resulted in an inhibition of DNA binding (Trautwein et al., 1994), whereas phosphorylation at Ser105 by protein kinase C within the activation domain of C/EBP- β enhances its transcriptional efficacy (Trautwein et al., 1993). To identify the region that is important for gene activation by

phosphorylation of Ser105 of C/EBP- β , a series of C/EBP- β mutants were constructed, and domain swapping experiments with the DNA-binding domain of GAL4 were performed. These experiments point to an acidic region located between amino acids 21 and 105 of C/EBP- β that activates genes independently of the DNA-binding domain and the leucine zipper of C/EBP- β . Computer-assisted predictions revealed two regions, a helical and a hydrophobic region in the transactivation domain, that could be important in mediating the direct interaction with the basal machinery. Site-directed mutagenesis of acidic residues in both regions demonstrates that the hydrophobic region located between amino acids 85 and 95 is the likely motif for the interaction with the basal machinery. These results demonstrated that a hydrophobic region in the acidic transactivation domain of C/EBP- β seems to be relevant in mediating gene activation of C/EBP- β -dependent genes (Trautwein et al., 1995).

9. *Phosphorylation of C/EBP- β Ser239 leads to C/EBP- β Nuclear Export.* Tumor necrosis- α (TNF- α) treatment of primary mouse hepatocytes or TNF- α overexpression in a mouse model of cachexia induces oxidative stress, nitric oxide synthase expression, and phosphorylation of C/EBP- β on Ser239 within the nuclear localization signal, thus inducing its nuclear export, which inhibits transcription from the albumin gene. Similar molecular abnormalities were found in the liver of patients with cancer-related cachexia. (Buck et al., 2001b). The cytoplasmic localization and association of phosphorylated C/EBP- β Ser239 with CRM1 (exportin-1) in TNF- α -treated hepatocytes were shown to be inhibited by leptomycin B, a blocker of CRM1 activity. Hepatic cells expressing the nonphosphorylatable C/EBP- β alanine mutant were refractory to the inhibitory effects of TNF- α on albumin transcription since the mutant remained localized to the nucleus. Treatment of TNF- α mice with antioxidants or nitric oxide synthase inhibitors prevented phosphorylation of C/EBP- β on Ser239 and its nuclear export and rescued the abnormal albumin gene expression in these animals (Buck et al., 2001b).

10. *Phosphorylation of C/EBP- β Thr217 by p90 Ribosomal S Kinase.* The hepatotoxin CCl₄ was shown to be able to induce p90 ribosomal S kinase (RSK) leading to phosphorylation of C/EBP- β on Thr217 with consecutive stellate cell proliferation in normal mice. In contrast, CCl₄ treatment of C/EBP- β knockout mice (C/EBP- β ^{-/-}) or transgenic mice carrying the C/EBP- β -Ala217 mutant (a dominant-negative nonphosphorylatable mutant) led to stellate cell apoptosis. It could be demonstrated that the association of C/EBP- β phosphorylated at Thr217 with the procaspases 1 and 8 inhibited their activation and thus created a functional XEXD caspase inhibitory box in vivo and in vitro, which might be critical for cell survival (Buck et al., 2001a).

D. C/EBP- γ (Also Called Ig/EBP)

Analysis of cDNA and genomic clones shows that the murine C/EBP- γ gene encodes a small protein with a predicted molecular weight of 16.4 kDa that contains C/EBP family basic and leucine zipper domains but lacks the transcriptional activation domains present in C/EBP- α and C/EBP- β . In transfection assays, C/EBP- γ is neither an activator nor a repressor of transcription; however, C/EBP- γ inhibits the transcriptional ability of C/EBP- α and C/EBP- β , acting as a transdominant negative regulator. Thus, C/EBP- γ resembles LIP, another aforementioned negative regulator of the C/EBP family, in both structure and transcriptional activity (Cooper et al., 1995).

Biochemical fractionation and antibody supershift assays demonstrated that a preferred C/EBP- β heterodimeric partner is C/EBP- γ . Interestingly, C/EBP- γ was an ineffective repressor in HepG2 hepatoma cells despite the formation of C/EBP heterodimers, and C/EBP- α was not effectively inhibited in either intestinal endocrine L cells or HepG2 cells, whereas in all other cells types tested, C/EBP- γ acted as a transdominant negative regulator. These findings demonstrate that C/EBP- γ modulates C/EBP activity in a cell- and isoform-specific manner (Parkin et al., 2002). Although C/EBP- γ might play an important role as a transdominant negative regulator for the expression of many genes, the data in the literature on C/EBP- γ are exceptionally rare.

1. *Structure and Chromosomal Location of C/EBP- γ .* Human C/EBP- γ has been mapped to chromosome 19, while in the zebrafish C/EBP- γ was mapped to linkage group 7, which is syntenic with human chromosome 19. These findings extended synteny between linkage group 7 and human chromosome 19. In addition, these syntenies between zebrafish and human chromosomes are also conserved in the mouse genome (Lyons et al., 2001). As with the murine C/EBP- γ homolog, the avian C/EBP- γ gene comprises two exons, with the open reading frame encoded in exon 2. The 150-amino acid C/EBP- γ protein is highly conserved, as the avian protein shows more than 80% identity with the murine and human homologs (Davydov et al., 1995; Baglia et al., 1997).

E. C/EBP- δ

The C/EBP- δ transcription factor is known to be rarely expressed but sharply induced at an early stage of the acute phase response (Yamada et al., 1997).

1. *Chromosomal Localization.* By fluorescence in situ hybridization, using the biotinylated genomic clone as a probe, the C/EBP- δ gene was assigned to the pericentromeric region of human chromosome 8, most probably to 8q11 (Cleutjens et al., 1993).

2. *Transcriptional Induction of C/EBP- δ by Interleukin 6.* In contrast to C/EBP- β , whose activity in Hep3B

cells is modulated by IL-6 via a post-translational mechanism, C/EBP- δ is transcriptionally induced by IL-6. Another contrasting feature is that the C/EBP- δ cDNA transfected in Hep3B cells activates transcription from an IL-6 response element synthetic promoter in a constitutive manner that is not further enhanced by IL-6 (Ramji et al., 1993).

Treatment of HepG2 cells with IL-6 leads to rapid induction of C/EBP- δ mRNA. Transfection and gel shift analyses identified a binding site for the acute phase response factor/signal transducers and activators of transcription (APRF/STAT-3). These findings strongly indicate that C/EBP- δ gene expression is mediated by APRF/STAT-3 after IL-6 receptor-mediated phosphorylation (Yamada et al., 1997).

3. *Regulation of C/EBP- δ by Phosphorylation.* Several lines of evidence suggest that C/EBP- δ is regulated by phosphorylation and, in conjunction with C/EBP- β , is one of the major proteins responsible for the increased transcription of the SAA gene in response to inflammatory stimuli (Ray and Ray, 1994).

4. *Autoregulation of C/EBP- δ .* Binding analysis of two downstream binding sites of the C/EBP- δ gene revealed that both of these sites bound recombinant C/EBP- δ protein. Cotransfection analysis identified these sites as the *cis* elements for C/EBP- δ autoregulation (Yamada et al., 1998). It can thus be proposed that the C/EBP- δ gene is activated by APRF/STAT-3, and the expression level is then maintained by an autoregulation mechanism.

5. *Inhibition of C/EBP- δ Gene Expression by C/EBP- β and C/EBP- ζ .* Transfection and DNA binding analyses revealed that LIP, the shorter isoform of C/EBP- β , as well as C/EBP- ζ (synonyms: GADD153 or CHOP-10) were found to inhibit C/EBP- δ gene expression. DNA binding analysis has further indicated that both LIP and C/EBP- ζ form heterodimers with C/EBP- δ and inhibit the binding of C/EBP- δ homodimer to the C/EBP- δ binding sites located downstream of the C/EBP- δ gene. Taken together, these findings indicate that the maintained expression of the C/EBP- δ gene by autoregulation is inhibited and decreased to the basal level as a result of the competition of other C/EBP family proteins (Tanabe et al., 2000).

F. C/EBP- ζ (Also Called GADD153 or CHOP-10)

In most cells, one effect of DNA damage is a transient inhibition of DNA synthesis and cell growth. Five genes have been isolated that are induced during growth arrest by serum reduction, medium depletion, contact inhibition, or a 24-h exposure to hydroxyurea. The genes coding for these transcripts have been designated GADD (growth arrest and DNA damage inducible) (Fornace et al., 1989; Zhang et al., 1999). GADD153 is a ubiquitously expressed member of the C/EBP family of transcription factors and is induced by a wide variety of growth-

arresting and DNA-damaging agents. Under the current nomenclature, GADD153 is named C/EBP- ζ .

1. *C/EBP- ζ as a Dominant Negative Regulator of C/EBPs.* Functionally, C/EBP- ζ has been postulated to act as a dominant-negative regulator of C/EBPs. C/EBP- ζ encodes a C/EBP-related protein that lacks a functional DNA-binding domain. Since the C/EBP- ζ protein is capable of heterodimerizing with other C/EBPs, C/EBP- ζ may function as a negative regulator of these transcription factors (Carlson et al., 1993).

2. *Chromosomal Localization and Characteristics of the Promoter of C/EBP- ζ .* C/EBP- ζ is localized in the region 12q13.1-q13.2 on human chromosome 12. Sequence analysis indicated that the human promoter region is relatively G+C-rich and contains putative binding sites for multiple transcription factors, including recognition sites for TATA- and CAAT-binding proteins, six Sp1-binding sites, an activator protein-1 binding site, an E-26-specific sequence-binding protein-1 DNA-binding site, and four interleukin 6 response elements (Park et al., 1992).

3. *Regulation of C/EBP- ζ in Response to Oxidative Stress and DNA Damage.* The C/EBP- ζ promoter is strongly activated by methyl methanesulfonate, hydrogen peroxide, UV irradiation, and other DNA-damaging agents, but not by growth arrest signals. This suggests that separate and very different regulatory pathways are involved in the induction of the C/EBP- ζ gene by growth cessation and DNA damage (Luethy et al., 1990). It was demonstrated that glucose deprivation and leucine limitation markedly induce GADD153 mRNA levels in cell lines such as HeLa and 3T3-L1 and that addition of D-(+)-glucose or leucine, respectively, result in a rapid decrease of C/EBP- ζ mRNA. Similar induction and reversal of C/EBP- ζ expression were observed at the protein level. (Carlson et al., 1993; Bruhat et al., 1997).

Both promoter-deletion analysis and point mutation of an AP-1 site in an otherwise intact promoter support a significant role for AP-1 in transcriptional activation of C/EBP- ζ by UV-C or oxidant treatment. It was shown that exposure of cells to oxidants or UV-C stimulates binding of Fos and Jun heterodimers to the C/EBP- ζ AP-1 element (Guyton et al., 1996).

It was demonstrated that treatment of HeLa cells with the calcium ionophores A23187 and ionomycin leads to the induction of C/EBP- ζ mRNA. The induction was rapid; increases in mRNA were detected after 90 min of treatment and near-maximum levels were achieved within 5 h of exposure to A23187. Elevated mRNA levels resulted from both an increase in the rate of C/EBP- ζ transcription and an increase in the stability of the C/EBP- ζ mRNA (Bartlett et al., 1992).

In contrast to the transcriptional activation of c-jun and collagenase in response to DNA damage, C/EBP- ζ induction involves neither protein kinase C nor tyrosine kinases but does appear to require p38 mitogen-acti-

vated protein kinase (MAP kinase) (Wang and Ron, 1996). Elevation of intracellular glutathione levels by treatment with *N*-acetylcysteine did not affect the methyl methanesulfonate-induced expression of the C/EBP- ζ gene, although it did diminish cadmium chloride-induced expression. These findings suggest that oxidative stress and DNA damage regulate C/EBP- ζ transcription through different pathways (Luethy and Holbrook, 1994).

4. *Phosphorylation of C/EBP- ζ* . C/EBP- ζ undergoes inducible phosphorylation on two adjacent serine residues (78 and 81). In vitro, C/EBP- ζ is phosphorylated on these residues by p38 mitogen-activated protein kinase (MAP kinase). A specific inhibitor of p38 MAP kinase, SB203580, abolished the stress-inducible in vivo phosphorylation of C/EBP- ζ . Phosphorylation of C/EBP- ζ on these residues enhanced its ability to function as a transcriptional activator and was also required for the full inhibitory effect of C/EBP- ζ on adipose cell differentiation. C/EBP- ζ thus serves as a link between a specific stress-activated protein kinase, p38, and cellular growth and differentiation (Wang and Ron, 1996).

5. *Autoregulation of GADD153 Expression*. It could be demonstrated that overexpression of C/EBP- ζ inhibits the transactivation of the C/EBP- ζ promoter by C/EBP- β . These findings provide evidence for an autoregulatory loop in which stress-induced C/EBP- ζ feeds back to attenuate C/EBP- ζ expression during the cellular response to stress (Fawcett et al., 1996).

6. *Differential Regulation of C/EBP- ζ Expression by Activating Transcription Factors 3 and 4*. ATF-3, a stress-inducible transcriptional repressor, is negatively regulated by C/EBP- ζ . Additionally, ATF-3 may repress the expression of its own inhibitor, C/EBP- ζ , and C/EBP- ζ mRNA is not present in situations when ATF-3 is induced, e.g., after intoxication of the liver by CC14. The C/EBP- ζ promoter contains two functionally important binding sites for ATF-3: an AP-1 site and a C/EBP-ATF composite site. The absence of either site reduces the ability of ATF-3 to repress the promoter (Wolfgang et al., 1997). Interestingly, ATF-4 activates, whereas ATF-3 represses the C/EBP- ζ promoter activity through the C/EBP-ATF composite site. ATF-3 also represses ATF-4-mediated transactivation and arsenite-induced activation of the C/EBP- ζ promoter. Numerous members of the CREB/ATF family are involved in the cellular stress response and regulation of stress-induced biphasic C/EBP- ζ expression in PC12 cells that involve the ordered, sequential binding of multiple transcription factor complexes to the C/EBP-ATF composite site (Fawcett et al., 1999).

7. *C/EBP- ζ -C/EBP- β Heterodimer-Mediated Gene Regulation*. A target gene that is positively regulated by GADD153-C/EBP heterodimers encodes murine carbonic anhydrase VI (CA-VI). The stress-inducible form of the gene is expressed from an internal promoter and encodes a novel intracellular form of what is normally a

secreted protein. Stress-induced expression of CA-VI is both GADD153- and C/EBP- β -dependent in that it does not occur in cells deficient in either gene (Sok et al., 1999). A GADD153 responsive element was mapped to the inducible CA-VI promoter, and in vitro footprinting revealed binding of GADD153-C/EBP heterodimers to that site. Rescue of CA-VI expression in C/EBP- $\beta^{-/-}$ cells by exogenous C/EBP- β and LIP suggests that the role of the C/EBP partner is limited to targeting the GADD153-containing heterodimer to the response element. This points to a pre-eminent role for GADD153 in CA-VI induction during stress (Sok et al., 1999).

G. Hormones, Metabolic Hepatic Functions, and the C/EBPs

1. *C/EBP- α and Energy Metabolism*. There is overwhelming evidence for C/EBP- α to play a central regulatory role in energy metabolism in the liver (Crosson et al., 1997). High levels of C/EBP- α mRNA were observed in tissues known to metabolize lipid and cholesterol-related compounds at uncommonly high rates and included liver, fat, intestine, lung, adrenal gland, and placenta. C/EBP- α is essential for adipogenesis and neonatal gluconeogenesis, as shown by the C/EBP- α knockout mouse (Arizmendi et al., 1999).

The effect of hormones and diabetes on C/EBP- α expression in rat H4IIE hepatoma cells and in rat liver was examined. Treatment of H4IIE cells with the glucocorticoid dexamethasone led to a 3-fold increase in C/EBP- α mRNA within 4 h. Insulin treatment produced a biphasic response, initially reducing mRNA levels up to the 4-h time point, but after 8 h a 2-fold increase in C/EBP- α mRNA was observed. Treatment with 8-chlorophenylthio-cAMP produced a 2-fold induction of C/EBP- α mRNA after 8 h. Western blot analysis indicated that the changes in mRNA in response to hormonal treatment generally resulted in corresponding alterations in C/EBP- α protein levels. An inhibition of C/EBP- α gene expression can be achieved in streptozotocin-diabetic rat liver, reflected by a decrease in both mRNA and protein levels that were partially reversed by insulin treatment. These results indicate that the expression of C/EBP- α in the liver is under complex control by both hormonal and metabolic signals, which is consistent with its role as a transregulator of genes that play a role in energy metabolism (Crosson et al., 1997) (see also Fig. 4).

2. *C/EBP- α in Gluconeogenesis and Detoxification of Ammonia and Bilirubin*. Ammonia produced by amino acid metabolism is detoxified through conversion into urea by the ornithine cycle in the liver, whereas carbon skeletons of amino acids are converted to glucose by gluconeogenic enzymes. Promoter and enhancer sequences of several genes for ornithine cycle enzymes interact with members of the C/EBP transcription factor family. Disruption of the C/EBP- α gene in mice causes hypoglycemia associated with the impaired expression

of gluconeogenic enzymes. In the livers of C/EBP- α -deficient mice, mRNA levels for the first (carbamoylphosphate synthetase), third (argininosuccinate synthetase), fourth (argininosuccinate lyase), and fifth enzyme (arginase) of five enzymes in the ornithine cycle were decreased. Protein levels for the first, second (ornithine transcarbamylase), fourth, and fifth enzymes were also decreased. Blood ammonia concentrations in the mutant mice were severalfold higher than in wild-type mice. Thus, C/EBP- α is crucial for ammonia detoxification by ornithine cycle enzymes and for coordination of gluconeogenesis and urea synthesis (Kimura et al., 1998) (see also Fig. 3).

It was previously reported that mice carrying a homozygous null mutation at the C/EBP- α locus died as neonates due to the absence of hepatic glycogen and resulting hypoglycemia. However, the lethal phenotype precluded further analysis of the role of *c/ebp- α* in hepatic gene regulation in adult mice. To circumvent this problem, a conditional knockout allele of *c/ebp- α* using the Cre/loxP recombination system was employed by Lee et al. (1997a). Homozygous *c/ebp-loxP* mice, *c/ebp- α (fl/fl)*, flanked by loxP sites were found to be indistinguishable from their wild-type counterparts. However, when Cre recombinase was delivered to hepatocytes of adult *c/ebp- α (fl/fl)* mice by infusion of a recombinant adenovirus carrying the cre gene, more than 80% of the *c/ebp- α (fl/fl)* genes were deleted specifically in liver, and C/EBP- α expression was reduced by 90%. This condition also resulted in a reduced level of bilirubin UDP-glucuronosyltransferase expression in the liver. After several days, the knockout mice developed severe jaundice due to an increase in unconjugated serum bilirubin. The expression of genes encoding phosphoenolpyruvate carboxykinase, glycogen synthase, and factor IX was also strongly reduced in adult conditional-knockout animals, whereas the expression of transferrin, apolipoprotein B, and insulin-like growth factor I genes was not affected. These results establish C/EBP- α as an essential transcriptional regulator of genes encoding enzymes involved in bilirubin detoxification and gluconeogenesis in adult mouse liver (Lee et al., 1997a).

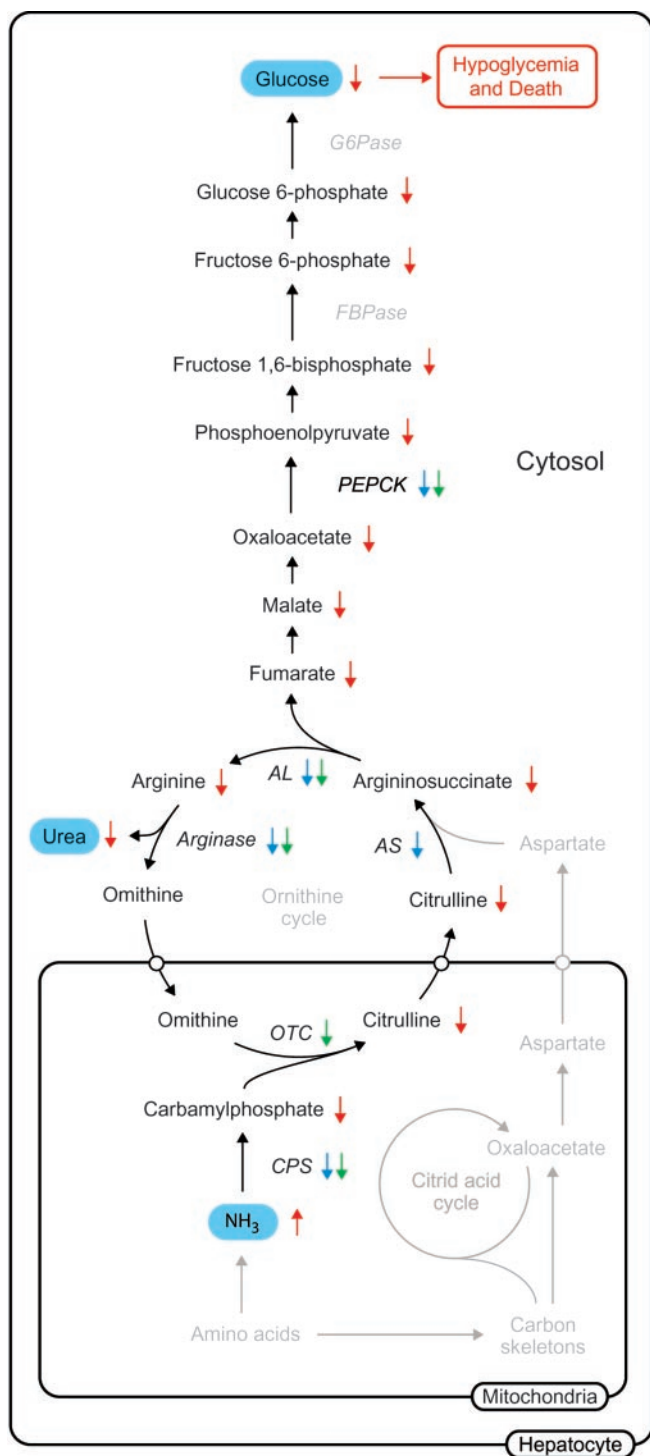
PEPCK is the rate-limiting enzyme of gluconeogenesis, and most, if not all, of the regulation of its activity is exerted at the level of gene expression. A number of hormones regulate transcription of this gene in a defined, tissue-specific fashion. For example, cAMP strongly induces PEPCK gene transcription in liver but provides only a weak response in kidney (Roesler, 2000). Results from a number of different studies indicate that cAMP responsiveness of this gene is mediated by a cAMP response unit (CRU), consisting of five *cis* elements. All five sequences are required for maximal responsiveness, and, potentially, four of these are binding sites for a C/EBP (Crosson and Roesler, 2000; Roesler, 2000). Since C/EBP- α and C/EBP- β are liver-enriched transcription factors, this may provide the molecular

basis for the liver-specific responsiveness to cAMP (Roesler, 2000). A curiosity of this promoter is that one of the *cis* elements present in the CRU is a CRE, which typically acts as a binding site for CREB. However, the nonconsensual CRE in the PEPCK promoter also binds C/EBP proteins with high affinity, and C/EBP- α can functionally substitute for CREB in this cAMP response unit whereas C/EBP- β cannot (Crosson and Roesler, 2000). The available data suggest that the PEPCK promoter is effective in altered states of cAMP responsiveness, depending on which transcription factors occupy specific *cis* elements in the CRU (Roesler, 2000).

It was shown that the CREB-binding protein (CBP) is able to enhance the induction of PEPCK gene transcription by thyroid hormone and that CBP is associated with the PEPCK gene in vivo. These results indicated that both C/EBP proteins and CBP participate in the regulation of PEPCK gene transcription by thyroid hormone (Jurado et al., 2002).

3. C/EBP- β and Energy Metabolism. It was demonstrated that the C/EBP- β gene can be primarily induced by glucocorticoids and by glucagon (Matsuno et al., 1996) (see Fig. 4). C/EBP- β has been linked to the metabolic and gene regulatory responses to diabetes. C/EBP- β has also been implicated as an essential factor underlying glucocorticoid-dependent activation of PEPCK gene transcription in vivo. C/EBP- β binds with high affinity to several sequences of the PEPCK gene promoter, and C/EBP- β protein is increased 200% in the livers of streptozotocin-diabetic mice, concurrent with increased PEPCK mRNA (Arizmendi et al., 1999). Studies with mice that are heterozygous or homozygous for a null mutation of the gene for C/EBP- β revealed that C/EBP- β is not essential to basal PEPCK mRNA levels (Arizmendi et al., 1999; Roesler, 2000). However, in streptozotocin-diabetic rats, C/EBP- β deletion leads to delayed hyperglycemia with no increase of free fatty acids in the plasma, limited induction of PEPCK and glucose 6-phosphatase genes, and no increased gluconeogenesis rate. Electrophoretic mobility shift assay supershifts of transcription factor C/EBP- α , bound to CRE, P3I, and AF-2 sites of the PEPCK promoter, were not increased in diabetic *c/ebp β ^{-/-}* mouse liver nuclei, suggesting that C/EBP- α does not substitute for C/EBP- β in the diabetic response of liver gene transcription (Arizmendi et al., 1999).

4. Insulin and Glucocorticoids Regulate Gluconeogenesis via C/EBP- β Isoforms. Highly related insulin response sequences (IRSs) mediate effects of insulin on the expression of multiple genes in the liver, including insulin-like growth factor binding protein-1 (IGFBP-1) and PEPCK. Gel shift studies evidenced that oligonucleotide probes containing an IRS from the IGFBP-1 or PEPCK gene form a similar complex with hepatic nuclear proteins. Unlabeled competitors containing the IGFBP-1, PEPCK IRS, or a binding site for C/EBP proteins inhibited the formation of this complex. Antibody



- ↓ decrease in mRNA of the enzyme in C/EBP- α conditional knock-out mice
- ↓ decrease in protein of the enzyme in C/EBP- α conditional knock-out mice
- ↓ decrease of enzymatic product
- ↑ increase in ammonia (NH_3)

FIG. 3. The role of C/EBP- α in ammonia detoxification, gluconeogenesis, and urea synthesis is depicted on the basis of data found in mice with disruption of the C/EBP- α gene (C/EBP- α conditional knockout mice) (Kimura et al., 1998; Arizmendi et al., 1999; Roesler et al., 2000). Mice with a homozygous null mutation of the C/EBP- α gene die as neonates due to absence of hepatic glycogen and the resulting hypoglycemia. Abbreviations used: CPS, carbamoylphosphate synthetase; OTC, ornithine transcarbamylase; AS, argininosuccinate synthetase; AL, argininosuccinate lyase.

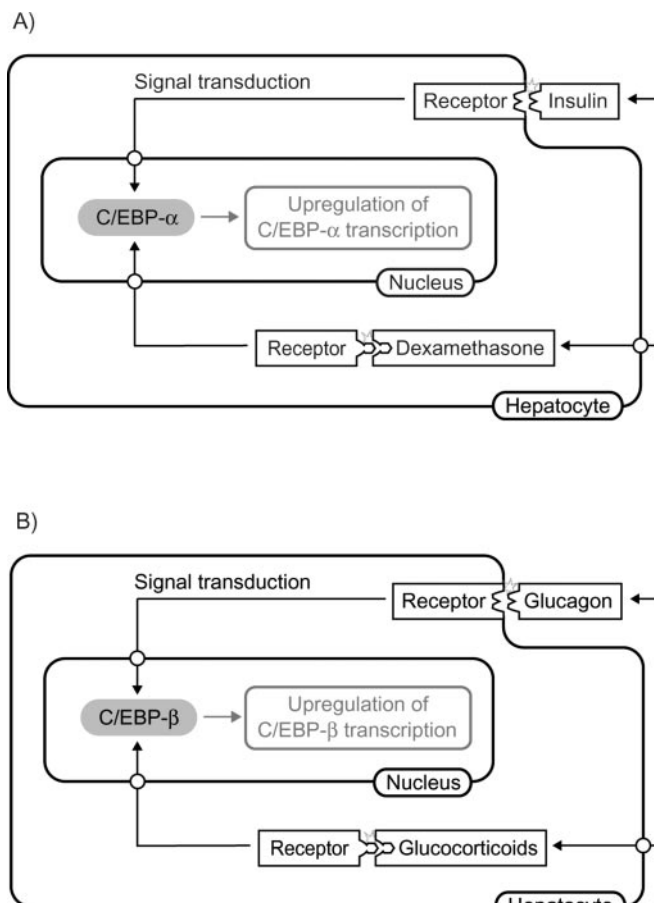


FIG. 4. A, the induction of the gene coding for C/EBP- α after insulin and/or dexamethasone exposure; B, the induction of the gene coding for C/EBP- β after glucagon and/or glucocorticoid exposure. C/EBP- α and C/EBP- β are involved in hepatic energy metabolism and are crucial for glucose homeostasis.

against C/EBP- β (but not other C/EBP proteins) supershifted this complex, and Western blotting of affinity-purified proteins confirmed that C/EBP- β is present in this complex. Studies with affinity purified and recombinant protein indicated that C/EBP- β does not interact directly with the IRS, but that other factors are required. Gel shift assays and reporter gene studies with constructs containing point mutations within the IRS revealed that the ability to interact with factors required for the formation of this complex correlated well with the ability of insulin to regulate promoter activity via this IRS ($r = 0.849$, $P < 0.01$). Replacing the IRS in reporter gene constructs with a C/EBP-binding site (but not an HNF-3/forkhead site or cAMP response element) maintained the effect of insulin on promoter activity (Ghosh et al., 2001). Together, these findings indicate that a nucleoprotein complex containing C/EBP- β interacts with IRSs from the IGF1BP-1 and PEPCK genes in a sequence-specific fashion and may contribute to the ability of insulin to regulate gene expression.

Insulin and glucocorticoids reciprocally regulate PEPCK expression primarily at the level of gene transcription. Indeed, it was demonstrated that glucocorti-

coids promote, whereas insulin disrupts, the association of CBP and RNA polymerase II with the hepatic PEPCK gene promoter in vivo. It was shown that accessory factors, such as the C/EBP- β isoform LAP, may recruit the coactivator CBP to drive transcription (Ghosh et al., 2001; Duong et al., 2002). Insulin increased protein levels of the C/EBP- β isoform LIP, an inhibitory form of C/EBP- β , using phosphatidylinositol-3-kinase-dependent intracellular signal transduction pathways. LIP concomitantly replaces liver-enriched transcriptional activator protein LAP on the PEPCK gene promoter, which can abrogate the recruitment of CBP and polymerase II, culminating in the repression of PEPCK expression and the attenuation of hepatocellular glucose production (Duong et al., 2002).

5. *Thyroid Hormone and Retinoic Acid Regulate C/EBP- α and - β in the Liver.* The effect of thyroid hormone and retinoic acid on the expression of C/EBP- α and - β was investigated in rat liver during development (Menendez-Hurtado et al., 1997). Congenital hypothyroidism caused a significant decrease in both C/EBP- α and C/EBP- β gene expression at early stages of postnatal development. This effect was tissue-specific, since thyroid hormone had no effect on C/EBP mRNA levels in brown fat. Injection of 15- and 30-day-old hypothyroid animals with thyroid hormone resulted in a slow recovery of hepatic C/EBP- α and - β mRNA levels. By comparison, retinoic acid was more rapid and potent in stimulating C/EBP- α and - β expression than thyroid hormone in hypothyroid animals (Menendez-Hurtado et al., 1997).

C/EBP- α and - β protein levels were markedly diminished in hypothyroid neonates, and the kinetics of induction of these proteins by thyroid hormone was faster than the one observed for the corresponding transcripts. The discrepancies observed between mRNA and protein levels suggest a translational or post-translational regulation of these genes as the major point of thyroid hormone action on these genes (Menendez-Hurtado et al., 1997).

6. *The C/EBPs and the Growth Hormone-Regulated Network of Transcription Factors.* Growth hormone (GH) regulates gene expression by modulating the concentration and/or activity of several transcription factors. The expression of HNF-6 is stimulated by growth hormone. Analysis of the promoter of HNF-6 showed that C/EBP- α binds to this promoter and inhibits its expression in protein-DNA interaction studies and in transfection experiments. This inhibitory effect involved an N-terminal subdomain of C/EBP- α and two sites in the promoter of HNF-6. Using liver nuclear extracts from hypophysectomized rats treated with growth hormone, evidence was obtained that demonstrated the role of growth hormone in inducing a rapid, transient decrease in the amount of C/EBP- α protein. This change induced by growth hormone coincides with a transient stimulatory effect of growth hormone on the expression

of HNF-6. Stimulation of the HNF-6 expression by growth hormone therefore involves lifting of the repression exerted by C/EBP- α in addition to the known stimulatory effects that are induced by growth hormone via STAT-5 (signal transducer and activator of transcription 5) and HNF-4 on that gene (Rastegar et al., 2000). In contrast to the above-mentioned in vivo studies, the results of experiments in primary rat hepatocyte cultures imply growth hormone mediated induction of C/EBP- α mRNA and protein expression and enhanced DNA binding of C/EBP- α to a consensus C/EBP-binding DNA oligonucleotide (Strand et al., 2000). These contradicting results of in vivo and in vitro studies may be a consequence to the nature of the consensus C/EBP-binding DNA oligonucleotide used, may reflect a more complex than anticipated effect of hypophysectomy, or may even point to a principal methodological problem associated with the extrapolation of results from cell culture experiments to the in vivo situation.

It could be demonstrated that C/EBP- β and - δ play a role in growth hormone-related induction of the c-fos promoter. Mutation of the c-fos promoter at the C/EBP binding site (CCAAT) lead to a 6- to 7-fold increase of the c-fos promoter activity after growth hormone stimulation, whereas the wild-type c-fos promoter shows only a 2-fold induction after growth hormone stimulation. It was concluded that C/EBPs restrain growth hormone-stimulated expression of c-fos (Liao et al., 1999). Electrophoretic mobility shift assays with nuclear extracts from 3T3-F442A fibroblast cells demonstrated that growth hormone stimulation led to increased binding of C/EBP- β and - δ to the c-fos C/EBP-binding site. Both LAP and LIP, forms of C/EBP- β , are detected in 3T3-F442A cells by immunoblotting. GH increases the binding of LAP/LAP and LAP/LIP dimers. Overexpression of LIP interferes with GH-promoted reporter expression in Chinese hamster ovary cells expressing GH receptors, consistent with the possibility that LIP restrains GH-stimulated c-fos expression. Overexpression of LAP elevates basal luciferase activity but does not influence promoter activation by GH, whereas overexpressed C/EBP- δ elevates basal promoter activity and enhances stimulation by GH. GH stimulates the expression of mRNA for C/EBP- β and - δ and increases levels of C/EBP- δ . Although C/EBP- β is not detectably altered, GH induces a shift to more rapidly migrating forms of LIP and LAP upon immunoblotting. Treatment of extracts from GH-treated cells with alkaline phosphatase causes a shift of the slower migrating form to the rapidly migrating form, consistent with GH promoting dephosphorylation of LIP and LAP. These studies implicate C/EBP- β and - δ in GH-regulated gene expression. They also indicate that GH stimulates the binding of C/EBP- β and - δ to the c-fos promoter and promotes the dephosphorylation of LIP and LAP. These events may contribute to the ability of C/EBP- β and - δ to regulate GH-stimulated expression of c-fos (Liao et al., 1999).

TABLE 1
An overview on experimental observations that demonstrate an involvement of C/EBPs in the transcriptional regulation of hepatic cytochrome P450 (CYP) genes

CYP Gene	Cell Cultures	Experimental Approach	Agonistic TFs	Antagonistic TFs	C/EBP-Binding	Authors
Human CYP2B6	Human HepG2 cells	Transfection with Zn-inducible C/EBP- α vector to achieve C/EBP- α reexpression	C/EBP- α			Jover et al., 1998
Human CYP2C9	Human HepG2 cells	Transfection with Zn-inducible C/EBP- α vector to achieve C/EBP- α reexpression	C/EBP- α			Jover et al., 1998
Human CYP2D6	Human HepG2 cells	Transfection with Zn-inducible C/EBP- α vector to achieve C/EBP- α reexpression	C/EBP- α			Jover et al., 1998
Rat CYP2C12 (female specific)	Primary rat hepatocytes	C/EBP- α overexpression in the presence and absence of IRE-ABP, and DNase I footprint analysis, gel mobility shift assays	C/EBP- α	IRE-ABP	nts -222 to -204	Buggs et al., 1998
Human CYP3A4	Human HepG2 cells	Cotransfection of C/EBP- α and DBP plus promoter deletion experiments with reporter gene assay	C/EBP- α		nts -57 to +11	Ourlin et al., 1997
Human CYP3A7	Human HepG2 cells	Cotransfection of C/EBP- α and DBP plus promoter deletion experiments with reporter gene assay	C/EBP- α		nts -55 to +13	Ourlin et al., 1997
Chicken CYP2H1	Chicken embryo hepatocytes, human HepG2 cells, COS-1 cells	Promotor deletion experiments and DNase I footprint analysis, gel mobility shift assays	Rat HNF-1 α , rat HNF-1 β , rat HNF-3 α or - β and chicken C/EBP- α	USF (repression, derepression by phenobarbital through competition at Barbie-box-like sequence)		Dogra and May, 1997
Rat CYP2B1	Human HepG2 cells	Promotor deletion experiments and DNase I footprint analysis, gel mobility shift assays	C/EBP- α		nts -64 to -45	Park and Kemper, 1996
Rat CYP2E1	Nuclear extracts from rat livers, primary rat hepatocytes	Promotor deletion experiments and DNase I footprint analysis, gel mobility shift assays, in vitro transfection assay	C/EBP- α		nts -66 to -42	Luc et al., 1996
Rat CYP2E2	Primary rat hepatocytes	Promotor deletion experiments and DNase I footprint analysis, gel mobility shift assays, in vitro transfection assay	C/EBP- α		nts -66 to -42	Luc et al., 1996
Rat CYP2C12	Primary rat hepatocytes	Transient transfection of a C/EBP- α expression vector plus promoter deletion experiments with reporter gene assay, DNase I footprint analysis, gel mobility supershift assays	C/EBP- α		nts -229 to -207	Tollet et al., 1995
Rat CYP2D5	Human HepG2 cells, <i>Drosophila melanogaster</i> cells	Transient transfection of a C/EBP- α or C/EBP- β or HNF-1 or a DBP expression vector plus promoter deletion experiments with reporter gene assay, DNase I footprint analysis, gel mobility supershift assays	Sp1, C/EBP- β forms a ternary complex with a possible agonistic protein-protein interaction Sp1-C/EBP- β			Lee et al., 1994a

IRE-ABP, insulin response element-A binding protein; TF, transcriptional factor; nts = nucleotides numbered in relation to the start site of transcription within the respective promoter of the CYP gene; agonistic TFs = transcription factors that enhance CYP gene expression; antagonistic TFs = transcription factors that inhibit CYP gene expression.

As discussed above, it could also be shown in GH-treated 3T3-F442A fibroblasts that GH is involved in the dephosphorylation of the C/EBP- β isoforms LIP and LAP (Piwien-Pilipuk et al., 2001). GH activates the Akt kinases and inhibits glycogen synthase kinase-3 (GSK-3). Phosphorylation studies using phosphatidylinositol 3'-kinase, alkaline phosphatase, Akt, and GSK-3 indicate that phosphatidylinositol 3'-kinase/Akt/GSK-3 mediates signaling between the growth hormone receptor and the nucleus, promoting dephosphorylation of C/EBP- β . Dephosphorylation increases binding of LAP complexes to the c-fos promoter and may contribute to the participation of C/EBP- β in GH-stimulated c-fos expression (Piwien-Pilipuk et al., 2001).

7. C/EBPs and the Control of Cytochrome P450 Gene Expression. Cytochromes P450 are a superfamily of heme proteins involved in oxidative metabolism of endogenous chemicals such as steroid hormones and human-made xenobiotics, including drugs and environmental pollutants. Hundreds of P450s have been demonstrated by cDNA and gene cloning in animals, plants, fungi, and bacteria. Most of the mammalian xenobiotic-metabolizing P450s, found within the eight subfamilies comprising the CYP2 family, are constitutively expressed in the liver. Transcriptional activation of individual P450 genes in the liver commences at distinct stages of development. Some P450 genes are preferentially expressed in one sex (for review, see Gonzalez and Lee, 1996). The mechanisms of liver-specific expression of the P450 genes are quite diverse. Recent studies have found that several different liver-enriched transcription factors including HNF-1 α , HNF-3, HNF-4, C/EBP- α , and C/EBP- β , and the more ubiquitously expressed factors Sp1, GABP- α/β , and NF2d9 are responsible for governing the transcription of P450 genes (for review, see Gonzalez and Lee, 1996; Jover et al., 1998).

In some cases, more than one factor can influence expression depending on the developmental stage of the animal, and ubiquitously expressed factors such as Sp1 have been found to cooperate with liver-enriched factors to maximally activate transcription of P450 genes. STAT protein- and phospholipase A₂-mediated signal transduction have also been implicated in sex-dependent expression of certain P450 genes (for review, see Gonzalez and Lee, 1996). Table 1 provides an overview on experimental observations that demonstrate an involvement of C/EBPs in the regulation of hepatic cytochrome P450 gene expression.

It could be demonstrated that intoxication of primary rat hepatocytes with Aroclor 1254, a complex mixture of polychlorinated biphenyls that is well known for its potency to induce drug-metabolizing enzymes, leads to highly significant and dose-dependent increase of C/EBP- α (up to 62-fold), HNF-1 (up to 7-fold), and HNF-4 (up to 8-fold) expression, and 50- and 4-fold inductions of glutathione S-transferase α 2 and several P450 monooxygenases, respectively. Based on the

ethoxyresorufin-O-de-ethylase assay, the gene expression and enzyme activity for CYP1A1 were in good agreement, but similar correlations could not be obtained for other P450 isozymes. The simultaneous induction of liver-specific transcription factors and of several detoxifying enzymes may point to a coordinate genomic response in cultures of rat hepatocytes upon treatment with Aroclor 1254 (Borlak and Thum, 2001).

Cytochrome P450 (P450) activity is very low or even absent in human hepatomas, a phenomenon that is accompanied by low levels of some liver transcription factors, notably C/EBP- α . To investigate a possible link between this transcription factor and hepatic P450 expression, HepG2 cells have been stably transfected with a C/EBP- α vector containing a zinc-inducible metallothionein promoter. Expression of functional C/EBP- α up to liver levels concomitantly increased the mRNAs of several members of the CYP2 family (2B6, 2C9, and 2D6), suggesting that this transcription factor may play a relevant role in controlling the hepatic expression of P450 enzymes (Jover et al., 1998).

In primary rat hepatocytes, overexpression of an insulin response element-A binding protein, a member of the SRY family of high-mobility group proteins, inhibits C/EBP- α -mediated activation of the female-specific cytochrome P450 2C12 (CYP2C12) gene, but not the male-specific cytochrome P450 2C11 (CYP2C11) gene. Insulin response element-A binding protein and C/EBP- α have overlapping specificity for the C/EBP- α target site in the CYP2C12 promoter and compete for binding to CYP2C12 DNA in vitro. In contrast, insulin response element-A binding protein and C/EBP- α bind distinct sequences in the CYP2C11 promoter. SRY-like proteins can bind to a subset of sequences recognized by the C/EBP family of DNA-binding proteins and modulate gene transcription in a context-specific manner (Buggs et al., 1998).

Liver-specific transcription of the CYP2D5 gene occurs postnatally and is dictated by a proximal promoter element, called 2D5, that is composed of a binding site for Sp1 or a related factor and an adjacent cryptic C/EBP site. Only C/EBP- β is capable of stimulating the 2D5 promoter in HepG2 cells; other C/EBP subfamily members are not. Activation of the 2D5 promoter by C/EBP- β is completely dependent on the presence of the Sp1 site. Domain switch experiments revealed that C/EBP- β proteins containing either the leucine zipper or the activation domain of C/EBP- α are unable to stimulate the 2D5 promoter but are fully capable of transactivating an artificial promoter bearing a high-affinity C/EBP site. Thus, the leucine zipper and the activation domain of C/EBP- β are absolutely required to support transactivation of the 2D5 promoter. Analysis of C/EBP- β -deficient mice showed that mutant animals are defective in expression of a murine CYP2D5 homolog in hepatic cells, confirming the selective ability of C/EBP- β to activate this liver-specific P450 gene in vivo. These findings il-

TABLE 2
An overview on experimental observations that demonstrate an involvement of C/EBPs in the transcriptional regulation of several acute phase proteins (AP proteins)

AP Protein	Cell Cultures	Experimental Approach	Agonistic TFs	Antagonistic TFs	C/EBP-Binding	Authors
Factor VIII	Human HepG2 cells	LPS stimulation of cell culture, promoter deletion experiments with reporter gene assays	C/EBP- β , NF- κ B			Begbie et al., 2000
α 1-Acid-glycoprotein	Rat livers	LPS treatment of rats, nuclear extracts, gel shift assays	C/EBP- α , C/EBP- β 35 kDa C/EBP- β isoform NF- κ B and Nopp140 as coactivators for C/EBP- β via protein-protein interactions Agonistic protein-protein interactions between C/EBP- β , Nopp140 and TFIIB Co-operative protein-protein interaction of NF- κ B p65 with C/EBP- β results in synergistic transcriptional activation of the SAA2 promoter AP-1, C/EBP- β	Nucleolin Antagonistic protein-protein interactions between hnRNP and C/EBP- β , interruption of agonistic Nopp140/C/EBP- β interactions	Acute phase response element (APRE) Distal regulatory element (DRE)	Rabek and Papaconstantinou, 1999 Petrovic et al., 1996 Yang et al., 1994 Lee et al., 1996 Miau et al., 1997, 1998 Xia et al., 1997
Serum amyloid A2 (SAA2)	Human hepatoma cell line	IL-1 β , IL-6, TNF- α and dexamethasone stimulation of cell culture, promoter deletion experiments with reporter gene assays				Kirschning et al., 1997
LPS binding protein (LBP)	Human HepG2 cells	Promoter deletion experiments with reporter gene assays, gel mobility assays of nuclear extracts	NF- κ B, C/EBP- β : both bind to overlapping nucleotides in a mutually exclusive manner C/EBP- β is a less potent transactivator than NF- κ B C/EBP- α , C/EBP- β , C/EBP- δ		Acute phase response element (APRE) in the multithormone-inducible enhancer nts -615 to -440 nts -53 to -40	Brasier et al., 1994 Boffa et al., 2002
Thrombin-activable fibrinolysis inhibitor (TAFI)	Human HepG2 cells					

TF, transcription factor; nts, nucleotides numbered in relation to the start site of transcription within the respective promoter of the acute phase protein gene; agonistic TFs, transcription factors that enhance acute phase protein gene expression; antagonistic TFs, transcription factors that inhibit acute phase protein gene expression.

illustrate that two members of a transcription factor family can achieve distinct target gene specificities through differential interactions with a cooperating Sp1 protein (Lee et al., 1997b).

H. The Role of C/EBPs in the Acute Phase Response

The acute phase response, an inflammatory process resulting from infection and/or tissue damage, is an early defense mechanism during which striking changes in protein synthesis occur mainly in the liver. The altered expression of many acute phase protein genes is regulated at the transcriptional level after hepatocyte stimulation by cytokines (e.g., IL-1 β , IL-6, TNF- α) and/or LPS. Some of these acute phase genes have DNA-binding sites for the C/EBP family of transcription factors (for review, see Koj, 1996) (Table 2). Figure 6 summarizes the events in the hepatocyte during the acute phase response after stimulation with the cytokines IL-1 β , IL-6, and TNF- α as detailed below.

1. *Differential Expression Patterns of the C/EBPs during the Acute Phase Response.* After intraperitoneal LPS administration, mice develop an acute phase response. In these mice, steady-state mRNA levels of C/EBP- α decrease significantly in the liver, lung, and fat tissues, and in vitro transcription assays indicate a decrease in the rate of C/EBP- α gene transcription in isolated liver nuclei upon LPS treatment. The steady-state levels of C/EBP- β and C/EBP- δ are massively increased within 4 h after LPS treatment (Alam et al., 1992). Likewise, burn injury leads to the induction of an acute phase response. It was shown that the expression of C/EBP- α decreases whereas C/EBP- β and C/EBP- δ mRNA levels increase (Gilpin et al., 1996). Furthermore, protein levels and DNA-binding activities of the 35- and 20-kDa C/EBP- β isoforms increase, whereas protein levels of the 42-kDa C/EBP- α decrease and the 30-kDa C/EBP- α remain high (Yiangou et al., 1998). These and similar observations lead to the conclusion that C/EBP- β and - δ are the primary mediators of the acute phase response.

Like C/EBP- β and C/EBP- δ , C/EBP- ζ mRNA is markedly induced in livers of rats treated with intraperitoneal LPS to initiate the acute phase response. Interestingly, its induction is temporally delayed relative to that of C/EBP- β and C/EBP- δ but is similar to that of acute phase reactants shown to be regulated by C/EBPs. It was shown that the C/EBP- ζ gene is itself regulated by C/EBP- β in inflammatory processes and that C/EBP- ζ is likely to contribute to the regulation of other genes whose expression is altered during the acute phase response (Sylvester et al., 1994).

2. *Differential Expression of C/EBP- α and C/EBP- β Isoforms.* The pool levels of the 42- and 30-kDa isoforms of C/EBP- α were high in control nuclear extracts and decreased significantly in LPS-treated rats. The binding activity and protein levels of the 20-kDa isoform of C/EBP- α were low in controls and increased dramati-

cally after LPS treatment (single intraperitoneal injection with 10 μ g of LPS in pyrogen-free saline). C/EBP- β isoforms with molecular masses of 35, 20, and 16 kDa were also detected. The 35-kDa pool level did not change, whereas the 20-kDa isoform was strongly induced in response to LPS. Western and DNA-protein binding analyses showed that p42 C/EBP- α forms specific complexes with the α 1-acid glycoprotein oligonucleotide in control nuclear extract and that p20 C/EBP- β forms complexes in LPS-treated liver. This suggests that synthesis of specific C/EBP- α and C/EBP- β isoforms occurred in the normal liver in vivo and that LPS mediated a differential initiation and inhibition of translation at specific AUG sites within each mRNA. The qualitative and quantitative changes in C/EBP- α and C/EBP- β isoform pool levels suggest that LPS or an LPS-stimulated factor can regulate the selection of AUG start sites for both activation and repression of translation. This regulation appears to involve an LPS-mediated down-regulation of initiation at the first AUG codon of the 42-kDa C/EBP- α and dramatic translational up-regulation at the fifth AUG codon of the 20-kDa C/EBP- α and the third AUG codon of the 20-kDa C/EBP- β . These regulatory events suggest the existence of proteins that may act as translational transacting factors (An et al., 1996).

3. *Acute Phase Response-Related mRNA/Protein Interaction leads to Liver-Enriched Transcriptional Inhibitory Protein Translation.* LPS-induced acute phase response in mouse livers leads to an elevated expression of the low-molecular-weight C/EBP- β isoform, LIP (Welm et al., 2000) (see also Fig. 5). The 5' region of C/EBP- β mRNA has been shown to be involved in the regulation of LIP translation. It could be demonstrated that binding of cytoplasmic proteins to the 5' region of C/EBP- β mRNA is altered in response to LPS administration. One of the major changes is induced binding of a cytoplasmic protein that is immunologically identical to the previously characterized RNA-binding protein CUGBP1 (Timchenko et al., 1999a; Welm et al., 2000). Induction of CUGBP1 binding activity in liver cytoplasm during the acute phase response is accompanied by the elevation of CUGBP1 binding activity on polysomes. CUGBP1 immunoprecipitated from livers of LPS-treated mice, but not from normal animals, is capable of inducing LIP translation in a cell-free translation system. The ability of CUGBP1 to induce LIP translation during the acute phase response depends on phosphorylation of CUGBP1 (Welm et al., 2000).

4. *Increased Liver-Enriched Transcriptional Inhibitory Protein Translation Leads to Reduced C/EBP- α mRNA Levels.* The elevated LIP translation during the acute phase response as well as after partial hepatectomy leads to increased binding of LIP to the C/EBP consensus site found within the mouse C/EBP- α promoter. This binding correlates with the reduction of C/EBP- α mRNA levels in both biological situations. Co-transfection experiments showed that full-length

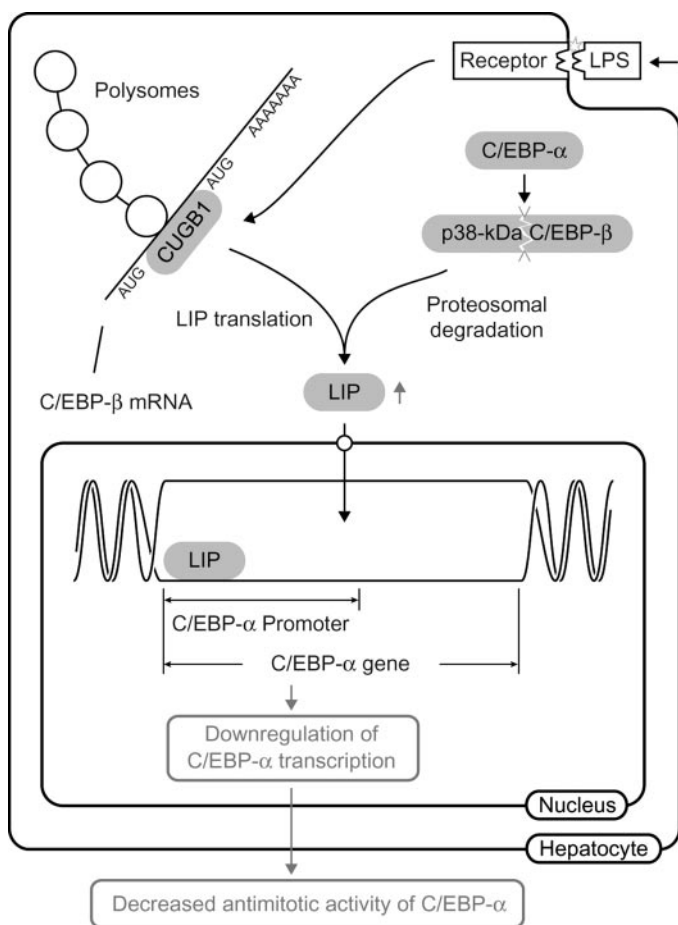


FIG. 5. After LPS administration, binding of phosphorylated CUGP1 to the 5' region of C/EBP- β mRNA leads to translation of the shorter LIP isoform during the acute phase response (based on data from Timchenko et al., 1999a; Welm et al., 2000). LIP can be produced by two mechanisms: alternative translation and a proteolytic cleavage of full-length C/EBP- β . In C/EBP- α knockout mice (C/EBP- $\alpha^{-/-}$), the regulation of C/EBP- β proteosomal degradation is impaired (based on data from Burgess-Beusse et al., 1999; Welm et al., 1999), whereas the induction of C/EBP- α in cultured cells leads to induced cleavage of C/EBP- β to generate the LIP isoform. Increased LIP levels lead to decreased C/EBP- α expression. This mechanism constitutes an alternative indirect autoregulatory loop for the regulation of C/EBP- α expression. For further details see text.

C/EBP- β activates the C/EBP- α promoter, whereas LIP blocks this activation. These data suggest that the dominant negative isoform of C/EBP- β , LIP, down-regulates the C/EBP- α promoter in liver and in cultured hepatocytes. Because full-length C/EBP- α and C/EBP- β proteins regulate liver proliferation, this function of LIP may be important in liver growth and differentiation (Welm et al., 2000).

5. Tumor Necrosis Factor α -Mediated Post-translational Regulation of C/EBPs. The proinflammatory cytokine TNF- α initiates post-transcriptional activation of cytokine-inducible C/EBPs that identifies a mechanism that enables hepatocytes to respond immediately to inflammatory stress. Experimental evidence stems from cultured hepatocytes (SV40 tsA255 virus-transformed rat adult liver cell line RALA255-10G) in the presence of recombinant TNF- α or mutated TNF- α peptides that specifically activate either p55 or p75 TNF receptors.

Within 5 to 10 min after treatment of hepatocyte cultures with any of these agents, nuclear concentrations of C/EBP- β and C/EBP- δ double and remain 2- to 4-fold greater than control cultures for 30 min (Yin et al., 1996). Consistent with these results, gel mobility shift assays demonstrate a 3-fold increase in nuclear C/EBP- β - and C/EBP- δ -DNA binding activity in TNF- α -treated cells, and immunocytochemistry confirms rapid redistribution of these C/EBPs into the nucleus. In contrast, mRNA and whole cell protein concentrations of C/EBP- β and - δ are not altered by TNF- α exposure, and nuclear concentrations of another C/EBP isoform, C/EBP- α , are decreased by 80% (Yin et al., 1996) (see Fig. 6).

6. Interleukin 6-Mediated Post-translational Phosphorylation of C/EBP- β . IL-6 is a pleiotropic cytokine playing important roles in immunity, hemopoiesis, and inflammation. Both the DNA binding activity and the transactivating capacity of C/EBP- β are induced in hepatoma cells by treatment with IL-6 through a post-translational mechanism, implicating it as a nuclear target of IL-6 and as a mediator of the IL-6-dependent transcriptional activation of liver genes during the acute phase response (Poli et al., 1990). On the other hand, C/EBP- β is involved in the activation of the IL-6 promoter in response to IL-1 β and bacterial lipopolysaccharide. Under some conditions, the C/EBP- β gene is transcriptionally activated by IL-1 β and lipopolysaccharide, whereas in other instances, its binding to cognate DNA sequences is enhanced by cytokines (Akira et al., 1990). IL-6 signaling is known to involve the activation of two independent transcription factors: STAT-3 (through phosphorylation by Jak kinases) and C/EBP- β (through activation of the ras/raf pathway) (Koj, 1996) (see Fig. 6).

Experiments with IL-6-deficient (knockout) mice demonstrated that IL-6 is essential for the hepatic induction of acute phase mRNAs like α 1-acid glycoprotein and C-reactive protein upon localized tissue damage but not upon systemically induced inflammation. The defective mRNA induction of acute phase genes is paralleled by a defective activation of STAT-3, thus suggesting a direct relationship among IL-6 function, STAT-3 activation, and the induction of acute phase genes. On the other hand, it was demonstrated by making use of C/EBP- β -deficient (knockout) mice that the induction of IL-6 by a variety of stimuli does not necessarily require C/EBP- β activity in vivo. In contrast to the predicted activating role of C/EBP- β , IL-6 levels are increased in the C/EBP- β -deficient mice, suggesting that C/EBP- β may act as a modulator of the IL-6 gene in vivo. The generation of C/EBP- β , IL-6 double-mutant mice demonstrated that IL-6 hyperproduction is responsible for the development of Castleman's lymphoproliferative disease described in the C/EBP- β -deficient mice, since the disorder is completely blocked by inactivating the IL-6 gene (Alonzi et al., 1997).

7. Influence of Interleukin 6 on C/EBP- β Transcription. Transfection experiments using promoter con-

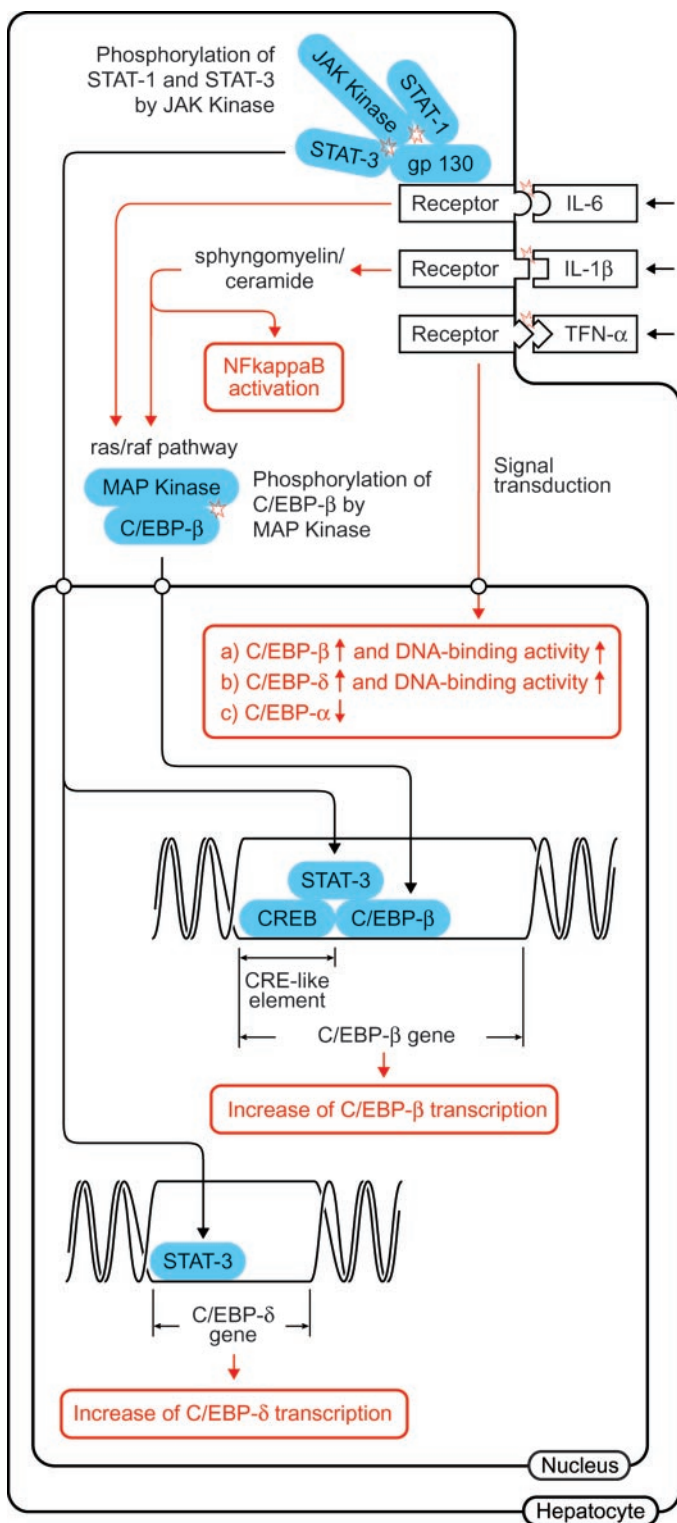


FIG. 6. The effects of IL-1 β , IL-6, and TNF- α stimulation on hepatocytes during the acute phase response are summarized schematically (for details see text). Red arrows depict consequences, whereas black arrows depict actual molecule translocations.

structs with mutated CRE-like elements could demonstrate that IL-6 controls C/EBP- β gene transcription via CRE-like elements in the C/EBP- β promoter without any STAT-3 DNA binding motifs (Niehof et al., 2001b)

(see Fig. 6). Luciferase reporter gene assays showed that STAT-3 activation through the glycoprotein 130 signal transducer molecule is involved in mediating IL-6-dependent C/EBP- β transcription. Southwestern analysis indicated that IL-6 induces binding of a 68-kDa protein to the recently characterized CRE-like elements in the C/EBP- β promoter. Further analysis using STAT-1/STAT-3 chimeras identified specific domains of the protein that are required for the IL-6-dependent increase in C/EBP- β gene transcription. Overexpression of the amino-terminal domain of STAT-3 blocked the IL-6-mediated response, suggesting that the STAT-3 amino terminus has an important function in IL-6-mediated transcription of the C/EBP- β gene (Niehof et al., 2001b). From these data it can be assumed that protein/protein interactions between STAT-3 and the protein complex at the CRE-like elements of the C/EBP- β promoter contribute significantly to the regulation of C/EBP- β transcription.

8. *Transcriptional Induction of C/EBP- δ after Interleukin 6 Stimulus.* C/EBP- δ is sharply induced at an early stage of the acute phase response (Yamada et al., 1997) (see Fig. 6). Treatment of HepG2 cells with IL-6 leads to rapid induction of C/EBP- δ mRNA. Transfection and gel shift assays lead to the identification of a binding site for APRF/STAT-3. These findings strongly suggest that C/EBP- δ gene expression is mediated by APRF/STAT-3 after IL-6 receptor-mediated phosphorylation (Yamada et al., 1997). Several lines of evidence suggest that C/EBP- δ is regulated by phosphorylation and, in conjunction with C/EBP- β , is one of the major proteins responsible for the increased transcription of the SAA gene in response to inflammatory stimuli (Ray and Ray, 1994).

9. *A Pivotal Role for C/EBP- α in the Acute Phase Response.* Studies with C/EBP- α knockout mice demonstrated that neonatal mice treated with purified bacterial lipopolysaccharide or recombinant IL-1 β resulted in a strong acute phase response in wild-type mice, but a response in C/EBP- α null animals was completely lacking. The C/EBP- α knockout and wild-type mice demonstrated elevations in C/EBP- β and - δ mRNA expression and DNA binding as well as increased DNA binding of NF- κ B, all of which are known to be important in the acute phase response. Null mice, however, failed to activate STAT-3 binding in response to lipopolysaccharide. A pivotal role for C/EBP- α in the induction of the acute phase response in vivo could therefore be established (Burgess-Beusse and Darlington, 1998).

10. *Interactions of C/EBP- α and C/EBP- β with the Nuclear Matrix.* The presence of C/EBP- α and C/EBP- β was established on the nuclear matrix. Their relative concentrations on the matrix reflected the developmental stage- and acute phase response-related fluctuations observed in the nuclear extract. Thus, they progressively increased as development proceeded, whereas during the acute phase response, C/EBP- α con-

centrations decreased and C/EBP- β concentrations increased. In addition, the levels of both transcription factors were always notably higher in the nuclear matrix than in the extracts (Dinic et al., 2000). The observed changes and overall enrichment of the nuclear matrix with regulatory proteins might reflect the importance of transcription factor/nuclear matrix interactions for the in vivo regulation of C/EBP- α and C/EBP- β functions in the acute phase response.

11. Protein/Protein Interaction between Nuclear Factor κ B p65 and C/EBP- β . The synergistic induction of the major acute phase reactant SAA2 expression by IL-1 β and IL-6 is mediated by NF- κ B and C/EBP- β . Electrophoretic mobility shift analysis indicated that NF- κ B p65 (RelA) and p50, but not p52 or c-Rel, bind specifically to the NF- κ B site of the SAA2 promoter in response to IL-1 stimulation. In addition, C/EBP- β and C/EBP- δ , but not C/EBP- α , bind specifically to the C/EBP site of SAA2 in response to IL-6 stimulation. Transient cotransfection analysis indicated that cooperative association of NF- κ B p65 with C/EBP- β and, in particular, with C/EBP- δ , results in synergistic transcriptional activation of the SAA2 promoter. When incubated together, NF- κ B p65 and C/EBP- β formed a ternary complex by direct protein/protein interaction. Mutational analysis demonstrated that the C terminus region of the Rel homology domain and the C terminus of the activation domain of p65 are important for its interaction with C/EBP- β (Xia et al., 1997).

12. Nucleolin Is an Antagonist to C/EBP- β in the Acute Phase Response. A transcription factor with a positive regulatory function, C/EBP- β , and a transcription factor with a negative regulatory function, factor B, have been identified as the two most important proteins responsible for the induction of the α -1 acid glycoprotein gene, a typical acute phase response gene. The purified factor B has been identified as nucleolin by amino acid sequence analysis. Biochemical and functional studies established further that nucleolin is a transcriptional repressor for α -1 acid glycoprotein and possibly other acute phase response genes. Thus, in addition to the many known functions of nucleolin, such as rRNA transcription, processing, ribosome biogenesis, and the shuttling of proteins between the cytoplasmic and nuclear compartments, it may also function as a transcriptional repressor (Yang et al., 1994).

13. Nuclear Factor κ B and Nopp140 Act as Coactivators for C/EBP- β . NF- κ B and a 140-kDa phosphoprotein named Nopp140 interact with different C/EBP- β isoforms that are activators for the α -1 acid glycoprotein gene. Known inducers of NF- κ B like IL-1 β and IL-6 can selectively activate the α -1 acid glycoprotein gene via NF- κ B as a coactivator for C/EBP- β (Lee et al., 1996). In addition to interacting with C/EBP- β , Nopp140 interacts specifically with TFIIB. Distinct regions of Nopp140 that interact with C/EBP- β and TFIIB have been characterized and the sequence of Nopp140 contains several

stretches of serine- and acidic amino acid-rich sequences, which are also found in ICP4 of herpes simplex virus type 1, a known transcription factor that interacts with TFIIB. The physical interaction between TFIIB and wild-type Nopp140 or several deletion mutants of Nopp140 correlates well with the ability of Nopp140 to activate the α -1 acid glycoprotein gene synergistically with C/EBP- β . Thus, the molecular mechanism for α -1 acid glycoprotein gene activation may involve the interaction of C/EBP- β and TFIIB mediated by the coactivator Nopp140 (Miau et al., 1997).

14. Heterogeneous Nuclear Ribonucleoprotein K as a Negative Regulator of C/EBP- β -Mediated Gene Activation. Physical and functional interactions between C/EBP- β and heterogeneous nuclear ribonucleoprotein (hnRNP) K result in the repression of C/EBP- β -dependent transactivation of the α -1 acid glycoprotein gene. Genomic footprinting assays indicate that hnRNP K cannot bind to the promoter region of α -1 acid glycoprotein gene or interfere with the binding of C/EBP- β to its cognate DNA site. Importantly, α -1 acid glycoprotein gene activation by the synergistic interaction of Nopp140 and C/EBP- β is abolished by hnRNP K. The kinetics of appearance of C/EBP- β -hnRNP K complex in the nuclear extract after initiation of the acute phase reaction indicates that hnRNP K functions as a negative regulator of C/EBP- β -mediated activation of the α -1 acid glycoprotein gene (Miau et al., 1998).

15. The Role of the Hypothalamic-Pituitary-Adrenal Axis in the Acute Phase Response. The regulatory effect of IL-1 β on the activation of C/EBP- α , C/EBP- β , and C/EBP- δ in hepatocytes of normal and adrenalectomized rats was examined in vivo. C/EBP- β and C/EBP- δ mRNA levels were enhanced by IL-1 β , whereas that of C/EBP- α was not affected by treatment with this interleukin in both normal and adrenalectomized rats. The magnitude of the induction was strikingly higher for C/EBP- δ in adrenalectomized animals, indicating a suppressive effect of corticosteroids in the IL-1 β regulatory pathway. For C/EBP- α , protein synthesis was higher than expected in IL-1 β -treated adrenalectomized animals compared with normal rats (Magalini et al., 1995).

The effects of either long-term absence of glucocorticoids (adrenalectomized rats treated with chronic release pellets of placebo) or extended exposure to pharmacologic levels of glucocorticoids (adrenalectomized rats treated with chronic release pellets of dexamethasone) on the expression of selected acute phase proteins and various members of the C/EBP family of transcription factors were examined. Both hypothalamic-pituitary-adrenal axis manipulations resulted in a reduction of the acute phase response as assessed by the LPS-mediated induction of acute phase proteins and C/EBP gene expression in rats, with dexamethasone treatment exhibiting a greater inhibitory effect than adrenalectomy alone. Induction of hemopexin, α -1 acid glycoprotein, α -2 macroglobulin, GADD153, C/EBP- β , and

C/EBP- δ mRNAs by LPS were all abolished in dexamethasone-treated rats (Eastman et al., 1996). These findings have direct implications for patients undergoing chronic high-dose glucocorticoid therapy after bilateral adrenalectomy, since they may not be able to produce an effective acute phase response as a consequence of diminished host defense mechanisms.

I. C/EBPs and Cell Cycle Control

1. *C/EBP- α Expression and Growth Arrest.* In fat and liver tissues, C/EBP- α expression is limited to fully differentiated cells (Birkenmeier et al., 1989; Umek et al., 1991). Dedifferentiated cells show decreased levels of C/EBP- α expression. Increased C/EBP- α expression, e.g., in a stable cell line or in the terminal phase of adipogenesis, is associated with proliferative growth arrest and a specialized cell phenotype (Umek et al., 1991; Runge et al., 1997). Experimental premature expression of C/EBP- α in adipoblasts causes a direct cessation of mitotic growth (Umek et al., 1991). Mice lacking C/EBP- α display hyperproliferation of alveolar type II cells, which implies C/EBP- α for a relevant role in controlled growth arrest of alveolar type II cells (Sugahara et al., 2001). The following sections condense our recent knowledge on C/EBP- α , C/EBP- β , and their role in cell cycle control in the liver. C/EBP- α is involved critically in diverse mechanisms to block hepatocyte proliferation. These mechanisms are summarized in Fig. 7 and discussed in some detail below.

2. *The Glucocorticoid-Induced G₁ Cell Cycle Arrest Is Mediated by C/EBP- α .* The glucocorticoid-stimulated expression of C/EBP- α is required for the steroid-mediated G₁ cell cycle arrest of minimal-deviation rat hepatoma cells. These cells include glucocorticoid-sensitive BDS1 cells and glucocorticoid-resistant EDR1 and EDR3 cells, which are epithelial tumor cells minimally derived from rat Reuber hepatoma (Cook et al., 1988). Comparison of C/EBP- α transcript and active protein levels induced by the synthetic glucocorticoid dexamethasone in glucocorticoid growth-suppressible (BDS1), nonsuppressible receptor-positive (EDR1), and nonsuppressible receptor-deficient (EDR3) hepatoma cell proliferative variants revealed that stimulation of C/EBP- α expression is a rapid, glucocorticoid receptor-mediated response, associated with the G₁ cell cycle arrest (Ramos et al., 1996). Consistent with the role of C/EBP- α as a critical intermediate in the growth suppression response, maximal induction of transcription factor mRNA occurred within 2 h of dexamethasone treatment, whereas maximal inhibition of [³H]thymidine incorporation was observed 24 h after steroid treatment (Ramos et al., 1996). As a direct functional approach, ablation of C/EBP- α protein expression and DNA binding activity by transfection of an antisense C/EBP- α expression vector blocked the dexamethasone-induced G₁ cell cycle arrest of hepatoma cells but did not alter general glucocorticoid responsiveness. Transforming growth factor

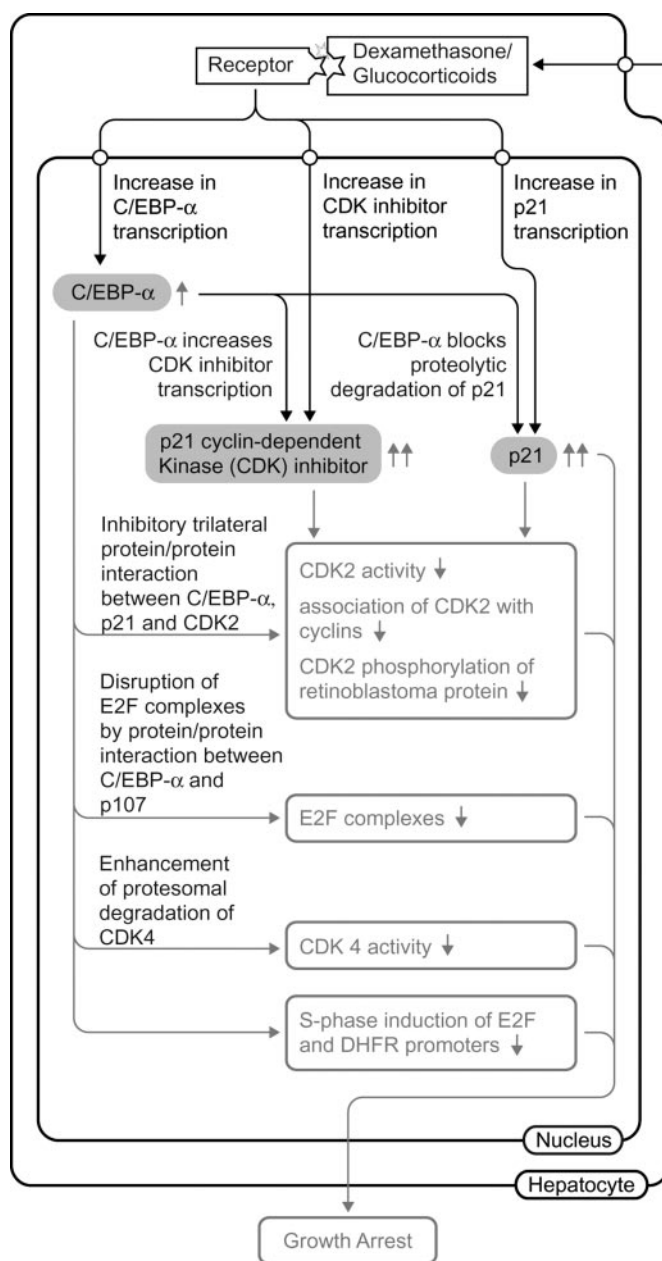


FIG. 7. This schematic representation summarizes the findings of Timchenko et al. (1996, 1997, 1998, 1999a,b), Cha et al. (1998), Cram et al. (1998), Slomiany et al. (2000), Harris et al. (2001), and Wang et al. (2001), which have shown that C/EBP- α blocks hepatocyte proliferation via diverse mechanisms. Furthermore, the effects of dexamethasone or glucocorticoid treatment that lead to growth arrest in hepatocytes are shown.

β (TGF- β) induced a G₁ cell cycle arrest in C/EBP- α antisense-transfected minimal-deviation rat hepatoma cells, demonstrating the specific involvement of C/EBP- α in the glucocorticoid growth suppression response (Ramos et al., 1996). Constitutive expression of a conditionally activated form of C/EBP- α caused a G₁ cell cycle arrest of BDS1 hepatoma cells in the absence of glucocorticoids. In contrast, overexpression of C/EBP- β or C/EBP- δ had no effect on hepatoma cell growth. Taken collectively, these results demonstrate that the steroid-induced expression of C/EBP- α is necessary to

mediate the glucocorticoid G_1 cell cycle arrest of rat hepatoma cells and suggest a role for this transcription factor in the growth control of liver-derived epithelial tumor cells (Ramos et al., 1996).

In BDS1 rat hepatoma cells, the synthetic glucocorticoid dexamethasone stimulated a rapid and selective increase in expression of the p21 cyclin-dependent kinase (CDK) inhibitor mRNA and protein and virtually abolished CDK2 phosphorylation of the retinoblastoma protein. A glucocorticoid responsive region within the promoter of the p21 CDK inhibitor gene could be identified that contains a putative DNA-binding site for the transcription factor C/EBP- α (Cha et al., 1998).

Wild-type rat glucocorticoid growth-suppressible BDS1 hepatoma cells as well as hepatoma cells, which express antisense sequences to C/EBP- α and ablate its protein production (as4 cells), were used to investigate the role of this transcription factor in the glucocorticoid regulation of p21 gene expression. The stimulation of p21 protein levels and promoter activity as well as inhibition of CDK2-mediated retinoblastoma protein phosphorylation by the synthetic glucocorticoid dexamethasone required the expression of C/EBP- α . Overexpression of C/EBP- α in as4 cells rescued the dexamethasone responsiveness of the p21 promoter. Site-directed mutagenesis of the p21 promoter revealed that dexamethasone stimulation of p21 promoter activity required the C/EBP consensus DNA-binding site. Furthermore, in glucocorticoid receptor-defective EDR1 hepatoma cells, dexamethasone failed to stimulate C/EBP- α and p21 protein expression and promoter activities. These results have established a functional link between the glucocorticoid receptor signaling pathway that mediates a G_1 cell cycle arrest of rat hepatoma cells and the transcriptional control of p21 by a cascade that requires the steroid-regulated induction of C/EBP- α gene expression (Cram et al., 1998) (see Fig. 7).

3. *Protein/Protein Interactions among p21, cdk2, cdk4, and C/EBP- α .* C/EBP- α is expressed at high levels in quiescent (nondividing) hepatocytes and in differentiated adipocytes. In hepatocytes, C/EBP- α has a dominant antiproliferative function but must interact with other factors to regulate hepatocyte-specific gene expression (Diehl et al., 1996). Deletion and cotransfection experiments with human C/EBP- α demonstrated that only one of both intact activation domains AD1 (amino acids 84 to 116) or AD2 (amino acids 154 to 245) is required for antimitotic activity in Hep3B2 cells (hepatoma cell line) (Hendricks-Taylor and Darlington, 1995).

In cultured HT1 cells (a mouse preadipocyte cell line that contains the human C/EBP- α gene under Lac repressor control), C/EBP- α inhibits cell proliferation by increasing p21 gene expression and by post-translational stabilization of p21 protein (also called WAF-1/CIP-1/SDI-1). Furthermore, induction of p21 is responsible for the ability of C/EBP- α to inhibit proliferation, because transcription of antisense p21 mRNA elimi-

nates growth inhibition by C/EBP- α (Timchenko et al., 1996) (see Fig. 7).

In C/EBP- α knockout newborn mice, p21 protein levels are reduced in the liver, and the fraction of hepatocytes synthesizing DNA is increased. More than 30% of the hepatocytes in C/EBP- α knockout animals continue to proliferate at day 17 of postnatal life when cell division in wild-type littermates is low (3%). p21 protein levels are relatively high in wild-type neonates but undetectable in C/EBP- α knockout mice (Timchenko et al., 1997). The reduction of p21 protein in the highly proliferating livers that lack C/EBP- α suggests that p21 is responsible for C/EBP- α -mediated control of liver proliferation in newborn mice (Timchenko et al., 1997). During rat liver regeneration after partial hepatectomy, the amounts of both C/EBP- α and p21 proteins are decreased before DNA synthesis (6 to 12 h) and then return to presurgery levels at 48 h (Timchenko et al., 1997).

Although C/EBP- α controls p21 protein levels, p21 mRNA is not influenced by C/EBP- α in the liver. Using coimmunoprecipitation and a two-hybrid assay system, an interaction of C/EBP- α with p21 proteins was demonstrated. Studies of p21 stability in liver nuclear extracts showed that C/EBP- α blocks proteolytic degradation of p21. This demonstrates that C/EBP- α regulates hepatocyte proliferation in newborn mice and that the level of p21 protein is under post-transcriptional control in the liver, consistent with the hypothesis that protein/protein interaction with C/EBP- α determines p21 levels (Timchenko et al., 1997). Two sites within the C/EBP- α protein could be identified that are able to interact with p21. One interaction site was localized to the leucine zipper region (amino acids 313–360), whereas the other site was found to reside between amino acids 119 and 226 in activation domain two (AD2) (Harris et al., 2001) (see Fig. 7).

It could be demonstrated that CDK2 also interacts with C/EBP- α . C/EBP- α can cooperate with p21 to inhibit CDK2 activity in vitro. Deletion experiments suggested that both the N- and C-terminal p21- and CDK2-binding sites are required for C/EBP- α to exert its effects on CDK2 and that a single binding site for p21 or CDK2 is not sufficient to cooperate in inhibiting CDK activity. C/EBP- α mutants incapable of inhibiting CDK2 activity in vitro do not inhibit proliferation in cultured cells. However, C/EBP- α mutants defective in DNA binding inhibit proliferation as effectively as wild-type protein. These findings show that C/EBP- α -mediated growth arrest occurs through protein interactions and is independent of its transcriptional activity (Harris et al., 2001) (see Fig. 7).

It could be shown that C/EBP- α directly interacts with cdk2 and cdk4 and arrests cell proliferation by inhibiting these kinases. A short-growth inhibitory region of C/EBP- α has been mapped between amino acids 175 and 187. This portion of C/EBP- α is responsible for direct

inhibition of cyclin-dependent kinases and causes growth arrest in cultured cells. C/EBP- α inhibits cdk2 activity by blocking the association of cdk2 with cyclins. Importantly, the activities of cdk4 and cdk2 are increased in C/EBP- α knockout livers, leading to increased proliferation. These data demonstrate that C/EBP- α brings about growth arrest through direct inhibition of cdk2 and cdk4 (Wang et al., 2001) (see Fig. 7).

It could be demonstrated that C/EBP- α enhances a proteasome-dependent degradation of cdk4 during growth arrest in the liver of newborn mice and in cultured cells. Overexpression of C/EBP- α in several biological systems leads to a reduction of cdk4 protein levels, but not mRNA levels. Experiments with several tissue culture models reveal that C/EBP- α enhances the formation of cdk4-ubiquitin conjugates and induces degradation of cdk4 through a proteasome-dependent pathway. As a result, the half-life of cdk4 is shorter, and protein levels of cdk4 are reduced in cells expressing C/EBP- α . Gel filtration analysis of cdk4 complexes shows that a chaperone complex cdk4-cdc37-Hsp90, which protects cdk4 from degradation, is abundant in proliferating livers that lack C/EBP- α , but this complex is weak or undetectable in livers expressing C/EBP- α . It could be demonstrated that C/EBP- α disrupts the cdk4-cdc37-Hsp90 complex via direct interaction with cdk4 and reduces protein levels of cdk4 by increasing proteasome-dependent degradation of cdk4 (Wang et al., 2002) (see Fig. 7).

4. C/EBP- α and p107 Protein/Protein Interaction Disrupts E2F Complexes. It was observed that C/EBP- α controls the composition of E2F complexes through interaction with the retinoblastoma-like protein p107 during prenatal liver development. S Phase-specific E2F complexes containing E2F, DP, cdk2, cyclin A, and p107 were observed in the developing liver. In wild-type animals, these complexes disappeared by day 18 of gestation and were no longer present in the newborn mice. In the C/EBP- α knockout mouse, the S phase-specific complexes did not diminish and persisted to birth. The elevation of levels of the S phase-specific E2F-p107 complexes in C/EBP- α knockout mice correlated with the increased expression of several E2F-dependent genes such as those that encode cyclin A, proliferating cell nuclear antigen, and p107. The C/EBP- α -mediated regulation of E2F binding is specific, since the deletion of another C/EBP family member, C/EBP- β , does not change the pattern of E2F binding during prenatal liver development. The addition of bacterially expressed, purified His-C/EBP- α to the E2F binding reaction resulted in the disruption of E2F complexes containing p107 in nuclear extracts from C/EBP- α knockout mouse livers. Ectopic expression of C/EBP- α in cultured cells also led to a reduction of E2F complexes containing retinoblastoma family proteins (Timchenko et al., 1999b). Coimmunoprecipitation analyses revealed an interaction of C/EBP- α with p107 but none with cdk2,

E2F1, or cyclin A. A region of C/EBP- α that has sequence similarity to E2F is sufficient for the disruption of the E2F/p107 complexes. Despite its role as a DNA-binding protein, C/EBP- α brings about a change in E2F complex composition through a protein/protein interaction. The disruption of E2F-p107 complexes correlates with C/EBP- α -mediated growth arrest of hepatocytes in newborn mice (Timchenko et al., 1999b) (see Fig. 7).

The inhibition of cell division by C/EBP- α in mouse L cells cannot be reversed by simian virus 40 T antigen, by oncogenic ras, or by adenovirus E1a protein. When expressed in thymidine kinase-deficient L cells or NIH 3T3 cells, C/EBP- α is detected in a protein complex that binds to the E2F binding sites found in the promoters of the genes for E2F-1 and dihydrofolate reductase. Bacterially expressed C/EBP- α has no affinity for these E2F sites, but when recombinant C/EBP- α is added to nuclear extracts from mouse fibroblasts, a new E2F binding activity appears, which contains the C/EBP- α protein. Using an E2F-DP1-responsive promoter linked to a reporter gene, it can be shown that C/EBP- α directly inhibits the induction of this promoter by E2F-DP1 in transient-transfection assays. Furthermore, C/EBP- α can be shown to inhibit the S phase induction of the E2F and dihydrofolate reductase promoters in permanent cell lines (Slomiany et al., 2000). These findings delineate a straightforward mechanism for C/EBP- α -mediated cell growth arrest through repression of E2F-DP1-mediated S phase transcription (see Fig. 7).

5. C/EBP- β Arrests the Cell Cycle before the G₁/S Boundary. During postnatal liver development, C/EBP- β expression and hepatocyte proliferation are mutually exclusive. In addition to transactivating liver-specific genes, C/EBP- β , but not C/EBP- α , arrests the cell cycle before the G₁/S boundary in HepG2 hepatoma cells. LIP, a liver inhibitory protein, which is translated from C/EBP- β mRNA lacking the activation domain of C/EBP- β , is not only ineffective in blocking hepatoma cell proliferation but also antagonizes the effect of C/EBP- β on the cell cycle. Deletion analysis indicated that this effect of LIP required only the DNA-binding and leucine zipper domains. In addition, it could be demonstrated that the integrity of the C/EBP- β dimerization and activation domains is indispensable for the arrest of cell proliferation induced by C/EBP- β (Buck et al., 1994). Thus, hepatocyte differentiation and its characteristic quiescent state may be modulated by the C/EBP- β LAP/LIP ratio.

6. Transforming Growth Factor α -Mediated Phosphorylation of C/EBP- β Leads to Hepatocyte Proliferation. TGF- α induces the activation of p90 RSK, which results in phosphorylation of rat C/EBP- β at Ser105 and of mouse C/EBP- β at Thr217, leading to stimulated proliferation of differentiated hepatocytes. It was demonstrated that this site-specific phosphorylation is essential for hepatocyte proliferation induced by TGF- α via RSK activation (Buck et al., 1999).

J. C/EBPS and Cellular Differentiation

1. *Transcription Factors during Liver Development and Oval Cell Differentiation.* The oval cells are thought to be the progeny of a liver stem cell compartment, and there is evidence to suggest that these cells participate in liver regeneration by differentiating into various hepatic lineages. To better understand the regulation of this process, the expression of liver-enriched transcriptional factors HNF-1 α , HNF-1 β , HNF-3 α , HNF-3 β , HNF-3 γ , HNF-4 α , C/EBP- α , C/EBP- β , and DBP was determined in an experimental model of oval cell proliferation and differentiation. The steady-state mRNA levels of HNF-1 α , HNF-3 α , HNF-4 α , and C/EBP- β gradually decreased during the late period of embryonic liver development, whereas HNF-1 β , HNF-3 β , and DBP increased. In the adult rat, liver expression of all liver-enriched transcription factors is restricted to the hepatocytes, but during early stages of oval cell proliferation both small and large bile ducts express HNF-1 α , HNF-1 β , HNF-3 γ , C/EBP- α , and DBP but not HNF-4 α . At later stages all of these factors are also expressed in proliferating oval cells. Expression of HNF-4 α was first observed when oval cells differentiate morphologically and functionally into hepatocytes and form basophilic foci. Based on these data, it was suggested that the up-regulation of "establishment" factors (HNF-1 and -3) may be an important step toward oval cell activation. The high levels of these factors in oval cells and embryonic hepatoblasts further substantiate the similarity between the two cell compartments. Furthermore, HNF-4 may be responsible for the final commitment of a small portion of the oval cells to differentiate into hepatocytes that form the basophilic foci and eventually regenerate the liver parenchyma (Nagy et al., 1994).

2. *Transdifferentiation of Pancreas to Liver and C/EBP- β Induction.* The appearance of hepatic foci in the pancreas has been described in animal experiments and in human pathology. It was demonstrated that pancreatic cells can be converted into hepatocytes by treatment with the synthetic glucocorticoid dexamethasone. This occurs both in a pancreatic cell line, named AR42J-B13, and in organ cultures of pancreatic buds from mouse embryos. Several features of the mechanism behind this transdifferentiation have been established. It was shown that a proportion of the hepatocytes arises directly from differentiated exocrine-like cells with no intervening cell division. This conversion is associated with induction of the transcription factor C/EBP- β and the activation of differentiated hepatic products. Transfection of C/EBP- β into the cells provoked transdifferentiation; conversely, a dominant-negative form of C/EBP- β can inhibit the process (Shen et al., 2000). These results indicate that C/EBP- β is a key component that distinguishes the liver and pancreatic programs of differentiation.

K. C/EBPs and Apoptosis

1. *Tumor Necrosis Factor α -Mediated Apoptosis and C/EBP- β .* Manganese superoxide dismutase (MnSOD), a TNF- α -inducible reactive oxygen-scavenging enzyme, protects cells from TNF- α -mediated apoptosis. Transient transfections of promoter-reporter gene constructs, in vitro DNA binding assays, and in vivo genomic footprint indicate that the murine MnSOD gene contains a 238-bp region in intron 2 that was responsive to TNF- α and IL-1 β . This TNF response element (TNFRE) had the properties of a traditional enhancer element that functioned in an orientation- and position-independent manner. In vivo genomic footprinting of the TNFRE revealed TNF- and IL-1-induced factor occupancy of sites that could bind NF- κ B and C/EBP. The 5' portion of the TNFRE bound C/EBP- β in vitro and was both necessary and sufficient for TNF responsiveness with the MnSOD promoter or with a heterologous promoter when in an upstream position. The 3' end of the TNFRE bound both NF- κ B and C/EBP but was not necessary for TNF responsiveness with the MnSOD promoter. However, this 3' portion of the TNFRE was required for the TNFRE to function as a downstream enhancer with a heterologous promoter. These data functionally separate the MnSOD TNFRE into a region responsible for TNF activation and another that mediates induction when it is downstream of a promoter (Jones et al., 1997). From these data it can be concluded that C/EBP- β displays antiapoptotic properties in connection with enhanced MnSOD expression after TNF- α stimulation.

2. *Fas-Induced Apoptosis and C/EBP- β .* Apoptotic cell death in the liver in response to activation of the Fas pathway has been implicated in human disease as well as liver remodeling and tissue repair. Differences in apoptotic cell death in the livers of C/EBP- β null mice using the Jo-2 agonistic anti-Fas antibody were investigated. Apoptotic injury was dramatically reduced in C/EBP- $\beta^{-/-}$ livers, as shown by a nearly 20-fold reduction in apoptotic hepatocytes 6 h post-Jo-2 treatment in C/EBP- $\beta^{-/-}$ hepatocytes compared with controls ($P < 0.04$) and reduced activation of caspase 3. Bid cleavage occurred in Jo-2-treated C/EBP- $\beta^{-/-}$ livers, indicating a block of Fas-induced injury distal to the death-inducing signaling complex. The level of the antiapoptotic protein bcl-x(L) was increased more than 10-fold in the mutant animals ($P < 0.04$), which can, at least in part, account for the protection from Fas-mediated apoptosis. In contrast, bcl-x(L) mRNA levels were unchanged. These observations link C/EBP- β to Fas-induced hepatocyte apoptosis through a mechanism that likely involves translational or post-translational regulation of bcl-x(L) (Mukherjee et al., 2001).

L. The Role of C/EBPs in Development

1. *Differential Regulation of C/EBPs and Their Isoforms during Development.* Specific C/EBPs are regulated differentially during the course of rat postnatal

liver development. During postnatal liver growth, several liver-specific functions emerge; this coincides with enhanced expression of C/EBP- α , - β , and - δ . Immediately after birth, nuclear expression of the 36-kDa C/EBP- δ protein increases, followed by increases in the 38-kDa C/EBP- β protein expression and enhanced expression of the 42-kDa C/EBP- α protein. Changes in C/EBP DNA binding activity accompany developmental increases in C/EBP proteins (for review, see Diehl et al., 1994). Messenger RNAs of C/EBP- α , C/EBP- β , and C/EBP- δ are also expressed by mature hepatocytes in primary cell culture from young adult rats (Borlak and Thum, 2001; Borlak et al., 2002). These results are consistent with the theory that variations in C/EBP expression and function help regulate hepatocyte terminal differentiation (for review, see Diehl et al., 1994).

2. *C/EBP- α and the Developmental Expression of Essential Metabolic Genes.* In liver and intestine C/EBP- α mRNA expression is coordinately induced just prior to birth (Birkenmeier et al., 1989). The role of C/EBP- α in the developmental expression of a subset of genes governing essential metabolic processes had been elucidated with a mutant mouse model that lacks C/EBP- α . The mutation resulted in the failure of the liver and white and brown adipose tissue to develop normal metabolic functions in the perinatal period, including hepatic glycogen synthesis and gluconeogenesis and the synthesis and deposition of triglyceride in adipose tissue (for review, see Darlington et al., 1995).

3. *Differential CYP3A7 and CYP3A4 Expression during Development.* In the human fetal liver, the cytochrome P450 enzyme CYP3A7 is expressed as early as the 13th week of gestation. Its expression continues into the perinatal period, but it is sharply repressed immediately after birth. Concomitantly, the expression of CYP3A4, not detectable in the fetus, sharply increases in the perinatal period to remain elevated throughout adulthood (Lacroix et al., 1997; review in Hakkola et al., 1998, 2001; Williams et al., 2002). It was demonstrated that CYP3A7 and CYP2A4 responded differently to C/EBP- α and DBP, two factors that exhibit a strict proliferation-dependent pattern of expression in the liver (Ourlin et al., 1997).

4. *Hepatic Expression of the Rat CYP2D5 Gene Is Regulated by C/EBP- β .* The rat CYP2D5 gene is expressed in liver cells. Its expression commences a few days after birth, and maximal mRNA levels are reached when animals reach puberty. The role of CYP2D5 in developmental programs was therefore studied. Transfection studies using a series of CYP2D5 upstream DNA chloramphenicol acetyltransferase gene fusion constructs identified a segment of DNA between nucleotides -55 and -156 that conferred transcriptional activity in HepG2 cells. Activity was markedly increased by cotransfection with a vector expressing C/EBP- β but was unaffected by vectors producing other liver-enriched transcription factors (C/EBP- α , HNF-1 α , and DBP).

DNase I footprinting revealed a region protected by both HepG2 and liver cell nuclear extracts between nucleotides -83 and -112. This region displayed some sequence similarity to the Sp1 consensus sequence and was able to bind the Sp1 protein, as assessed by a gel mobility shift assay. The role of Sp1 in CYP2D5 transcription was confirmed by transactivation of the 2D5-CAT construct in *D. melanogaster* cells by using an Sp1 expression vector. C/EBP- β alone was unable to directly bind the -83 to -112 region of the promoter but was able to produce a ternary complex when combined with HepG2 nuclear extracts or recombinant human Sp1. C/EBP- α was unable to substitute for C/EBP- β in forming this ternary complex. A poor C/EBP binding site is present adjacent to the Sp1 site, and mutagenesis of this site abolished formation of the ternary complex with the CYP2D5 regulatory region. These results established that both factors work in conjunction, possibly by protein/protein interaction, to activate the CYP2D5 gene (Lee et al., 1994a).

M. The Role of C/EBPs in Liver Regeneration

1. *Liver Regeneration after Concanavalin A-Induced Liver Injury.* Concanavalin A (Con A) injection into mice leads to immune-mediated liver injury. In Con A-induced liver injury, TNF- α - and IL-6-dependent signaling pathways known to be related to hepatocyte proliferation are activated (Trautwein et al., 1998). 5-Bromo-2'-deoxyuridine staining and nuclear cyclin A expression as markers of the S phase were first detected in hepatocyte nuclei 24 h after Con A injection, peaking after 48 h. An increase in TNF-dependent nuclear expression of the C/EBP- β isoform LAP was detected after 1 h, whereas an increase in RNA expression was evident only after 4 h. Expression of the C/EBP- β isoform LAP returned to normal before progression into the S phase. DNA binding of STAT-3 increased for up to 8 h. In addition to STAT-3, STAT-1 also binds to the same sequence, but DNA binding of the apoptosis-related STAT-1 started earlier than DNA binding of STAT-3, an important regulator of hepatocyte proliferation (Trautwein et al., 1998). These data demonstrated that after Con A insult, TNF- α - and IL-6-dependent signals probably trigger nuclear events regulating hepatocyte apoptosis and proliferation during liver injury.

2. *Hepatocyte Growth Factor Stimulates C/EBP- β and Nuclear Factor κ B Expression.* Hepatocyte growth factor (HGF) is an inducible cytokine that is essential for the normal growth and development of various tissues, such as the liver. HGF confers a major mitogenic stimulus to hepatocytes during liver regeneration (review in Matsumoto and Nakamura, 1992). HGF is a pleiotropic factor with mitogenic, morphogenic, mitogenic, cytotoxic, and/or growth-inhibitory activity. Although the signaling of HGF is mediated through the cell membrane receptor c-Met, the molecular mechanism of downstream signal transduction is, as yet, not fully un-

derstood. It was demonstrated that HGF stimulated the expression of C/EBP- β and NF- κ B, which are both key transcription factors responsible for the regulation of many genes under stress conditions or during the acute phase response. Biochemical and functional analysis evidenced a HGF-responsive element located in the region -376 to -352 (URE1) of the 5'-upstream regulatory sequence of the gene coding for C/EBP- β . Activation of NF- κ B by HGF was observed to precede the induction of C/EBP- β . Further studies indicate that NF- κ B can cooperate with C/EBP- β or other members of the C/EBP family to activate the C/EBP- β gene in both a URE1- and a URE2-dependent manner. These results suggest that induction of the C/EBP- β gene by HGF is mediated at least in part by the activation of NF- κ B. Activated NF- κ B then interacts with C/EBP- β , resulting in the induction of C/EBP- β (Shen et al., 1997).

3. *Hepatocyte Growth Factor Is Transactivated by C/EBP- β and C/EBP- δ .* A novel regulatory element, located between -6 and +7 bp (from the transcription start site) in the HGF basal promoter region was identified, which binds to inducible transcription factors and dictates responsiveness to extracellular stimuli that activate this gene. The core binding sequence for the inducible *cis*-acting factors was determined to be TTTGCAA (-4 to +3 bp) within the HGF promoter. Competition and gel mobility supershift assays showed that these binding complexes are composed of C/EBP- β and C/EBP- δ . DNA binding analysis also revealed that the binding site for the C/EBP family of transcription factors in the HGF promoter region overlaps that of another binding protein (complex C1), which binds specifically to a novel sequence with a core binding site of ACCGGT located adjacent to the C/EBP site (-9 to -4 bp). C1 binds to this region of the promoter and represses the inducible up-regulation by C/EBP through direct competition for their individual binding sites. Partial hepatectomy, which is known to activate HGF gene expression in the liver, increased C/EBP (especially C/EBP- β) binding activity to this region of the HGF promoter (Jiang and Zarnegar, 1997).

4. *Transcriptional Regulation of C/EBP- α in Liver Regeneration.* With Northern blots, the expression of C/EBP- α mRNA during liver regeneration and in cultures of primary rat hepatocytes was examined. C/EBP- α mRNA levels decreased to 60 to 80% within 1 to 3 h after partial hepatectomy, as hepatocytes moved from G₀ to G₁ and decreased further when cells progressed into S phase. In vitro transcription analysis was in agreement with the Northern blot data, thus suggesting that C/EBP- α is transcriptionally regulated in regenerating liver (Mischoulon et al., 1992).

5. *C/EBP- β Isoforms in Liver Regeneration.* The truncated C/EBP- β isoform LIP is induced in rat livers in response to partial hepatectomy via the alternative

translation mechanism (Burgess-Beusse et al., 1999; Timchenko et al., 1999a; Welm et al., 1999).

6. *C/EBP- α and C/EBP- β Expression after Partial Hepatectomy.* As mentioned above, expression of both C/EBP- α and C/EBP- β increased after partial hepatectomy. Steady-state levels of C/EBP- α mRNA increased 30% within 1 h of partial hepatectomy. This is followed by a transient increase in nuclear levels of C/EBP- α protein at 3 h after partial hepatectomy. Interestingly, increases in C/EBP- β mRNA and protein are more sustained. Levels of C/EBP- β mRNA increased 4- to 5-fold within 1 h of partial hepatectomy and remained increased throughout most of the prereplicative period. Nuclear levels of C/EBP- β protein are 2- to 3-fold greater than prehepatectomy levels at 3 to 6 h and do not approach basal levels until 24 h after partial hepatectomy. Gel mobility shift assays of nuclear extracts from regenerating livers indicated increases in nuclear protein expression that are associated with increased DNA binding of C/EBP- α -C/EBP- β heterodimers and C/EBP- β -C/EBP- β homodimers. These results demonstrate growth-related variations in the expression and DNA binding of both C/EBP- α and - β during liver regeneration and support the notion that altered C/EBP DNA binding may contribute to regeneration-associated changes in liver cell phenotype (Diehl and Yang, 1994).

After two-thirds partial hepatectomy, the liver is able to compensate for the acute loss of mass and maintains serum glucose levels and many of its differentiation-specific functions. When the interplay between differentiation and cell growth was studied during liver regeneration, the C/EBP- α protein level decreased more than 2-fold during the mid to late G₁ and S phase (8-24 h after hepatectomy) coordinately with a 3-fold increase in expression of C/EBP- β . Renormalization of the levels of these proteins occurred after the major proliferative phase. This inverse regulation of C/EBP- α and - β results in up to a 7-fold increase in the β/α DNA binding ratio between 3 and 24 h after hepatectomy that may have an important impact on target gene regulation. Nonetheless, total C/EBP binding activity in nuclear extracts remained relatively constant during the 7-day observation period after hepatectomy. Both C/EBP- α and - β are expressed in virtually all hepatocyte nuclei throughout the liver during the course of liver regeneration, and there is no exclusion of expression from hepatocytes that are expressing immediate-early gene products or undergoing DNA synthesis. The persistent expression of C/EBP- α and - β isoforms shows that C/EBP proteins contribute to the function of hepatocytes during physiologic growth and that significant amounts of these proteins do not inhibit progression of hepatocytes into S phase of the cell cycle despite the known antagonist functions of C/EBP- α and C/EBP- β (Greenbaum et al., 1995).

The rate of adult rat liver proliferation is normally low. It is markedly enhanced during compensatory re-

generation. Liver cell proliferation after partial hepatectomy, but not in response to chemical treatment with the antiandrogen cyproterone acetate, is associated with changes in C/EBP- α and C/EBP- β expression. This further supports the notion that changes in expression of transcription factors during liver growth in vivo are dependent on the growth inducer (Skrtic et al., 1997).

7. Influence of Age on Liver Regeneration. In old animals the regenerative response to partial hepatectomy is reduced and delayed (Timchenko et al., 1998). The expression of C/EBP- α in old rats (24 months) differed from its expression in young animals (6–10 months) during liver regeneration. Induction of proliferating cell nuclear antigen, a marker of DNA synthesis, occurred at 24 h after partial hepatectomy in young rats but was delayed and reduced in old animals. Induction of the mitotic-specific protein, cdc2 p34, is 3- to 4-fold less in regenerating liver of old rats than in the liver of young animals, confirming the reduced proliferative response in old animals. In young rats, the normal regenerative response involves a reduction of 3- to 4-fold in the levels of C/EBP- α protein at 3 to 24 h. In old animals, C/EBP- α is not reduced within 24 h after partial hepatectomy, but a decrease of C/EBP- α protein levels can be detected at 72 h after partial hepatectomy (Timchenko et al., 1998). Induction of C/EBP- β is also delayed in old animals. Changes in the expression of C/EBP proteins are accompanied by alteration of the CDK inhibitor p21, which is also decreased in young rats after partial hepatectomy but remains unchanged in old animals. High levels of p21 protein in older animals correlate with the lack of cdk2 activation. Possibly, the failure to reduce the amount of C/EBP- α and p21 is a critical event in the dysregulation of hepatocyte proliferation in old animals following partial hepatectomy (Timchenko et al., 1998).

8. Influence of Obstructive Jaundice on Liver Regeneration. During the process of liver regeneration C/EBP- α was found to stabilize p21(WAF1), whereas AP-1 and C/EBP- β enhance cyclin D1 and cyclin E expression, respectively. The differences in the expression of several transcription factors and cell cycle regulators were investigated between obstructive jaundiced and control rats before and after hepatectomy. The expressions and activities of C/EBP- α and - β were significantly decreased in the jaundiced group concomitant with a significantly lower cyclin E expression after hepatectomy as compared with controls. The activities of AP-1, cyclin D1, and p21(WAF1) were not significantly different between the two groups (Nakano et al., 2001). These results suggest that obstructive jaundice inhibits hepatic expression and activity of C/EBP- α and C/EBP- β , resulting in an impaired cyclin E expression that may be partly responsible for the frequently observed cell cycle dysfunction after hepatectomy in jaundiced patients. These findings may be clinically relevant for the failure of liver regeneration in patients with central bile duct carcinoma who underwent major hepatic resection.

9. Cyclooxygenase 2 Contributes to Liver Regeneration. Partial hepatectomy (PH) triggers a rapid regenerative response in the remaining tissue to reinstate organ function and cell mass. Among the molecules that change in the course of regeneration is an accumulation of prostaglandin E₂ in the sera of rats with PH. Analysis of the cyclooxygenase (COX) isoenzymes in the remnant liver showed the preferential expression of COX-2 in hepatocytes. Cultured regenerating hepatocytes expressed significantly higher levels of COX-2, a process that was not observed in the sham counterparts. Maximal expression of COX-2 was detected 16 h after PH with increased levels present even at 96 h. Pharmacological inhibition of COX-2 activity with NS398 shunted the up-regulation of cell proliferation after PH, which suggests a positive interaction of prostaglandins with the progression of the cell cycle. Similar results were obtained after PH of mice lacking the COX-2 gene. The expression of COX-2 in regenerating liver was concomitant with a decrease in C/EBP- α level and an increase in the expression of C/EBP- β and C/EBP- δ . These results suggest a contribution of the enhanced synthesis of prostaglandins to liver regeneration observed after PH (Casado et al., 2001). These findings could have potential clinical implications in patients undergoing major liver resection, since COX-2 inhibitors might be contraindicated in these patients in the postoperative course.

N. C/EBPs and their Role in Liver Tumor Biology

1. The Ratio of C/EBP- α and C/EBP- β Expression in Chemical Carcinogenesis. A silencer in the glutathione S-transferase P (GST-P) gene that is strongly and specifically expressed during chemical hepatocarcinogenesis has been identified. At least three transacting factors bind to multiple *cis* elements in the silencer. One of them, silencer factor B, is identical with C/EBP- β and binds to GST-P silencer 1 (GPS1). Many C/EBP- β binding sites are recognized by each of the C/EBP isoforms. Western blot analyses of C/EBP isoforms during chemical hepatocarcinogenesis revealed a decrease of C/EBP- α expression. However, there was no change in C/EBP- β level. In the nuclear extracts from normal liver, C/EBP- α was the dominant form that bound to GPS1, whereas both C/EBP- α and C/EBP- β bound to GPS1 in the nuclear extracts from carcinogenic liver. Furthermore, transfection assays showed that C/EBP- α not only repressed the GST-P promoter activity but also attenuated the transcriptional stimulation by C/EBP- β . These observations strongly suggest that the ratio of C/EBP- α to C/EBP- β is one of the important factors for the GST-P silencer activity, and the decrease of this ratio during hepatocarcinogenesis reduces the silencer activity and, consequently, increases the GST-P expression (Osada et al., 1995).

2. Protein/Protein Interaction between Mutant p53 and C/EBP- β in Liver Cancer. p53 is a tumor suppressor and transcription factor that is activated by genotoxic stress and mediates cell cycle arrest and apoptosis.

Overexpression of mutant p53, which is unable to mediate cell cycle arrest and/or apoptosis, is frequently found in undifferentiated hepatocellular carcinomas that typically display a lack of liver-specific gene expression (Tannapfel and Wittekind, 2002). It was demonstrated that wild-type p53 as well as tumor-derived p53 mutants repress C/EBP-mediated transactivation of the albumin promoter via a protein/protein interaction. Deletion analysis and domain swapping experiments showed that repression of C/EBP- β -mediated transactivation is dependent on the N-terminal domain of p53, the transactivation domain, the leucine zipper domain, and the inhibitory domain II (amino acids 163–191) of C/EBP- β (Kubicka et al., 1999).

3. Regulatory Role of Liver-Enriched Transcription Factors in Liver Cancer. In a Chinese expression profiling study of human hepatocellular carcinoma, the expression level of C/EBP- α was down-regulated in the tumor tissues as compared with normal liver tissue of the same patients, whereas HNF-1, HNF-3 β , HNF-4 α , and HNF-4 γ were up-regulated. These results suggested that liver-enriched transcription factors may play a regulatory role in human hepatocellular carcinoma (Xu et al., 2001). In another study from Japan, the comparison of the expression levels of the C/EBP- α gene in surgical specimens between hepatocellular carcinoma and nontumorous regions from the same patient revealed that in 9 of 13 cases, the expression level in the tumors was decreased compared with that in corresponding nontumorous regions (Tomizawa et al., 2002). Taken together, these data suggest that the expression of the C/EBP- α gene may be down-regulated in the majority of human hepatocellular carcinoma.

4. Repression of C/EBP- α -Mediated Transactivation by CAAT Displacement Protein in Human Liver Cancer? The observation that CDP can act as a competitive repressor for C/EBP- α -mediated transactivation (Antes et al., 2000) may indicate that CDP could play a significant, not yet described role in hepatocarcinogenesis, since the negative influence of C/EBP- α on cell cycle progression might be abolished when CDP expression is up-regulated. Repression by CDP involves competition for binding site occupancy and active repression via the recruitment of a histone deacetylase activity. CDP function is regulated by several post-translational modification events including phosphorylation, dephosphorylation, and acetylation (for review, see Nepveu, 2001).

There is further evidence that links CDP with cell cycle control, since it was demonstrated that CDP is a member of the CDP-cut/CDC2/cyclin A/pRB-complex (also called histone nuclear factor D) that influences the timing of cell cycle activation of the human histone H4 gene transcription at the G₁/S phase transition (Aziz et al., 1998; Last et al., 1999). Furthermore, it was shown that the phosphorylation of CDP by cyclin A-CDK1 contributed to reduced CDP activity as cells progress into the G₂ phase (Santaguida et al., 2001). Experimental

results support the notion that site-specific proteolysis of CDP generates a 110-kDa carboxyterminal peptide of CDP at the G₁/S phase transition of the cell cycle that displays an increased DNA binding activity to the ATC-GAT motif of CDP (Moon et al., 2001; for review, see Nepveu, 2001). Acetylation of CDP by p300/CREB-binding protein-associated factor was shown to be directed at conserved lysine residues near the homeodomain region and regulated CDP function. These observations are consistent with the ability of CDP to regulate genes as a transcriptional repressor, suggesting acetylation as a mechanism that regulates CDP function (Li et al., 2000). However, the importance of CDP phosphorylation or acetylation as well as site-specific proteolysis for repression of C/EBP- α -mediated transactivation by CDP in hepatocytes remains to be elucidated.

Human major histocompatibility class I antigen expression is important in controlling the metastatic growth of malignant tumors. Locus-specific down-regulation of major histocompatibility class I gene expression is frequently observed in human tumors, leading to decreased susceptibility to cytotoxic T-cell-mediated lysis. The identification of human CDP as a locus-specific repressor of HLA-B and C gene expression (Snyder et al., 2001) provided further clues that suggest that CDP may be an important player in human hepatocarcinogenesis. Further research on CDP might lead to targets for pharmacologic or gene therapeutic strategies against human hepatocellular carcinoma.

O. Human Disease with Causative C/EBP Involvement

1. Essential Hypertension in African-Americans. Hypertension is a serious health problem in Western societies, in particular for the African-American population. It has been long suggested that the angiotensinogen gene locus is involved in human essential hypertension. Ultimately, it could be shown that an A/G polymorphism at nucleotide position –217 in the promoter of the angiotensinogen gene plays a significant role in hypertension in African-Americans. The frequency of the –217A allele was increased significantly in African-American hypertensive subjects compared with normotensive controls. It could be demonstrated that the nucleotide sequence of this region of the angiotensinogen gene promoter was a strong C/EBP-binding site when nucleoside A was present at nucleotide position –217. Reporter constructs containing the human angiotensinogen gene promoter with nucleoside A at –217 had increased basal transcriptional activity upon transient transfection in HepG2 cells compared with reporter constructs with nucleoside G at position –217. Furthermore, IL-6 treatment in the presence or absence of overexpressed C/EBP- β increased the promoter activities of reporter constructs containing nucleoside A at –217, compared with reporter constructs containing nucleoside G at –217 (Jain et al., 2002). It was proposed that increased transcriptional activity of the –217A allele of the human

angiotensinogen gene is associated with hypertension in African-Americans.

III The D Site-Binding Protein

DBP (Mueller et al., 1990) is the founding member of the PAR family of bZip transcription factors. Other members of this family include thyroid embryonic factor (TEF) (Drolet et al., 1991), its avian ortholog vitellogenin promoter-binding protein (Iyer et al., 1991), and hepatic leukemia factor (HLF) (Hunger et al., 1992; Inaba et al., 1992). All of these proteins share high amino acid sequence similarities within an amino-terminal activation domain, a PAR domain rich in proline and acidic amino acid residues, and a carboxy-terminal moiety encompassing the bZip region necessary for DNA binding and dimerization. In vitro all PAR bZip proteins avidly bind the consensus DNA recognition sequence 5'-RTTAY.GTAAAY-3' as homo- or heterodimers (Falvey et al., 1996). DBP binds to similar sites on the promoters of albumin and cholesterol 7 α hydroxylase (CYP7A) genes as members of the C/EBP family of transcription factors. DBP as well as the C/EBPs recognize similar sequences with the core consensus binding sequences 5'RTTAY.GTAAAY3', with DBP being considerably less tolerant to deviations from the consensus site (Falvey et al., 1996). Interestingly, DBP as well as its mRNA accumulate to significant levels only in adult animals (Mueller et al., 1990). DBP is an important liver-enriched transcription factor that enables and influences tissue-specific transcriptional regulation of several genes (e.g., albumin, factor VIII). Furthermore, DBP is also involved in the circadian transcriptional regulation of several hepatic cytochrome P450 enzymes (P450 genes; e.g., CYP2C6). The following sections focus on the molecular and functional properties of DBP and its role in liver physiology.

A. Chromosomal Localization and Interspecies Conservation

The DBP gene was found to be syntenic both on human chromosome 19 and on rat chromosome 1. These results provide further evidence for conservation of synteny on these two chromosomes (and on mouse chromosome 7) (Szpirer et al., 1992). The human chromosomal DBP locus was assigned to chromosome 19q13 by a fluorescence in situ hybridization technique. This assignment was confirmed by means of human chromosome segregation in somatic cell hybrids (Khatib et al., 1994). Coding sequences of DBP, extending beyond the bZIP domain to the PAR region, were highly conserved in both human-human and interspecies comparisons. Conservation of the exon-intron boundaries of each bZIP domain encoding exon suggested derivation from a common ancestral gene. DBP mRNAs were expressed in all tissues and cell lines examined, including brain, lung, liver, spleen, and kidney (Khatib et al., 1994).

B. Genomic Structure of D Site-Binding Protein

Examination of the genomic structure of DBP revealed that the gene is divided into four exons and is contained within a relatively compact region of approximately 6 kilobases. These exons appear to correspond to functional divisions of the DBP protein. Exon 1 contains a long 5' untranslated region. Conservation between the rat and the human *DBP* genes with small open reading frames within exon 1 suggests that it may play a role in translational control. Exon 2 contains a limited region of similarity to the other PAR domain genes, which may be part of a potential activation domain. Exon 3 contains the PAR domain and differs by only 1 of 71 amino acids between rat and man. Exon 4, containing both the basic and the leucine zipper domains, is likewise highly conserved. The overall degree of homology between the rat and the human cDNA sequences is 82% for the nucleic acid sequence and 92% for the protein sequence (Shutler et al., 1996).

C. The Transactivation Domain of D Site-Binding Protein

It was demonstrated that DBP contains a 38-amino acid transactivation domain that is highly homologous to the transactivation domains of the transcription factors HLF and TEF. Deletion of this domain completely abrogated transcriptional activity of native DBP and GAL4-DBP fusion proteins. This domain functions as a modular transactivation domain that is a potent transcriptional activator when fused to the GAL4-DBD fusion protein. Although DBP itself is a liver-specific transactivator, the DBP transactivation domain is active in a variety of cell types, indicating that liver-specific activity is not an intrinsic property of this domain and must be conferred by other regions of the protein (Li and Hunger, 2001).

D. Cotranscriptional and Post-transcriptional Splicing of D Site-Binding Protein

Exon-intron boundaries were mapped on nascent RNA chains transcribed by RNA polymerase II. A fractionation method of nuclei into a chromatin pellet containing DNA, histones, and ternary transcription complexes and a supernatant containing the bulk of the nonhistone proteins and RNAs that are released from their DNA templates were developed. The transcripts of the gene encoding DBP recovered from the chromatin pellet and the supernatant were analyzed by S1 nuclease mapping. The large majority of the RNA molecules from the pellet appeared to be nascent transcripts, since, in contrast to the transcripts present in the supernatant, they were not cleaved at the polyadenylation site but rather contained heterogeneous 3' termini encompassing this site. Splicing intermediates were detected among nascent and released transcripts, suggesting that splicing occurs both cotranscriptionally and post-

transcriptionally. Furthermore, these experiments indicated that polyadenylation is not required for the splicing of the last DBP intron (Wuarin and Schibler, 1994).

E. The Proline and Acidic-Rich Domain and the p300 Coactivator Are Involved in Transactivation by D Site-Binding Protein

Experimental data suggest that p300 acts as a coactivator for DBP-mediated transactivation, since a 12 S E1A expression construct is able to disrupt DBP-mediated transactivation in HepG2 cells, whereas a p300 expression vector is able to restore DBP-mediated transactivation in contrast to a CBP vector (Lamprecht and Mueller, 1999). Deletion analysis of the DBP protein showed that the conserved PAR domain acts as a crucial part of an independent activation domain comprised of sequences in both the amino terminus and in the PAR domain of DBP when assayed in HepG2 cells. The coexpression of full-length expression constructs for both DBP and HLF resulted in a dramatic increase in activation mediated by GAL4-DBP fusion proteins, suggesting the involvement of a regulated coactivator in this process. Taken together, the PAR domain is required for DBP activation, which occurred through a p300-dependent process (Lamprecht and Mueller, 1999).

F. D Site-Binding Protein Forms Homo- and Heterodimers with Proline and Acidic-Rich Protein Family Members

DBP was shown to be able to form heterodimers with other family members of the proline and acidic-rich (PAR) protein family like HLF (Begbie et al., 1999). In transient transfection assays with HepG2 cells, HLF alone and in synergistic combination with the DBP was able to transactivate the promoters of the blood coagulation factors factor VIII and factor IX. At least some of the synergistic activation of the factor VIII promoter seen with HLF and DBP cotransfection could be attributed to increased binding of HLF-DBP heterodimers to two factor VIII promoter sites. Several observations indicated that the PAR family of transcription factors plays an important and complex role in regulating expression of the factor VIII and factor IX genes, involving the binding of both homodimeric and heterodimeric complexes of HLF and DBP to several sites in the promoters (Begbie et al., 1999).

G. The Rhythm of D Site-Binding Protein Expression Beats the Drum for Circadian Gene Regulation

In mammals, many physiological processes are subject to circadian regulation. These include sleep/wake cycles, body temperature, heartbeat, blood pressure, endocrine functions, renal activity, and liver metabolism (for review, see Schibler and Lavery, 1999). DBP accumulates in hepatocytes of adult rats according to a strictly controlled circadian rhythm (amplitude, approximately 1000-fold). In rat parenchymal hepatocytes, the

protein is barely detectable during the morning hours. At about 2 PM, DBP levels begin to rise, reach maximal levels at 8 PM, and decline sharply during the night. This rhythm persists with regard to its amplitude and phase in the absence of external time cues, such as daily dark/light switches (Wuarin and Schibler, 1990; Wuarin et al., 1992).

In rat and mouse liver the expression of all three related PAR bZIP proteins, TEF, HLF, and DBP, is subject to strong circadian regulation, peak and trough levels being reached in the early evening and morning, respectively (Wuarin and Schibler, 1990; Falvey et al., 1995; Fonjallaz et al., 1996; Lopez-Molina et al., 1997). In the case of DBP, the amplitude of circadian mRNA oscillation can largely account for the daily amplitude in protein oscillation (Fonjallaz et al., 1996). The mRNA accumulation oscillates not only in peripheral tissues such as liver, but also in neurons of the suprachiasmatic nucleus, believed to harbor the central circadian pacemaker (Ralph et al., 1990). According to the currently held model, these oscillators are synchronized via chemical cues by a master pacemaker, residing in the suprachiasmatic nucleus of the hypothalamus, which itself is entrained by the photoperiod (Yamazaki et al., 2000; for review, see Takahashi, 1995 and Hastings, 1997).

Although DBP is not essential for embryonic development and survival during adulthood, it is involved in the control of several circadian outputs. Mice homozygous for a *DBP* null allele differ from wild-type mice in the period length and the amplitude of circadian locomotor activity (Lopez-Molina et al., 1997), in several electroencephalogram parameters of sleeping behavior (Franken et al., 2000), and in the circadian expression of some liver genes, such as the ones specifying steroid 15 α hydroxylase (CYP2a4) and coumarin 7 hydroxylase (CYP2a5) (Lavery et al., 1999; for review, see Schibler and Lavery, 1999). Run-on experiments in isolated nuclei (Wuarin and Schibler, 1990; Lavery and Schibler, 1993) and physical mapping of nascent RNA chains (Wuarin and Schibler, 1990) suggest that circadian transcription plays a pivotal role in rhythmic DBP expression. Since *DBP*^{-/-} mice are still rhythmic and since DBP protein is not required for the circadian expression of its own gene, *DBP* is more likely to be a component of the circadian output pathway than a master gene of the clock (Lopez-Molina et al., 1997).

H. Regulation of the D Site-Binding Protein Promoter by CLOCK

Several lines of evidence indicate that circadian DBP transcription requires the basic helix-loop-helix-PAS (PER, ARNT, SIM) protein CLOCK, an essential component of the negative-feedback circuitry generating circadian oscillations in mammals and fruit flies (Antoch et al., 1997; King et al., 1997; Ripperger et al., 2000). Genetic and biochemical experiments suggest that CLOCK regulates DBP expression by binding to E-box motifs

within putative enhancer regions located in the first and second introns. DNase I mapping experiments in conjunction with two-dimensional electrophoretic mobility shift assays unveiled six putative regulatory sequences in the gene coding for DBP, of which two are located upstream (6 and 7) and four downstream (1–4) of the transcription initiation site. The sensitivity toward DNase I digestion of four of these regions (2, 4, 6, and 7) and that of the promoter region encompassing the transcription initiation site oscillate with the same phase as DBP transcription. Three of the putative regulatory sequences (2, 4, and 7) contain E-boxes that are potential binding sites for CLOCK. The presence of CLOCK-binding sites within DNase I-hypersensitive regions, which oscillate during the day in phase with DBP transcription, strongly suggested that CLOCK controls rhythmic hepatic DBP expression in a direct manner. It was therefore proposed that the regulation of DBP transcription is directly connected to the circadian feedback circuitry. DBP is rhythmically expressed in both suprachiasmatic nucleus neurons and most peripheral cell types and, as a transcription factor, can control the circadian expression of many target genes (Ripperger et al., 2000).

I. Changes of Feeding Times Influence Circadian D Site-Binding Protein Expression Patterns

In the liver, most known genes with rhythmic expression encode enzymes or regulatory proteins involved in food processing and energy homeostasis. These include cholesterol 7 α hydroxylase (CYP7) (Mitropoulos et al., 1972; Noshiro et al., 1990; Lavery and Schibler, 1993), the rate-limiting enzyme in the synthesis of bile acids, a number of cytochrome P450 enzymes involved in detoxification and elimination of food components (e.g., CYP2A5) (Lavery et al., 1999), enzymes involved in carbohydrate metabolism (e.g., PEPCK, glycogen synthase, glycogen phosphorylase) (Ishikawa and Shimazu, 1976; Roesler and Khandelwal, 1985; Frederiks et al., 1987), and transcription factors governing fatty acid metabolism (e.g., PPAR and spot 14) (Kinlaw et al., 1987; Lemberger et al., 1996). At least in the liver, the coordination of physiological needs during the absorptive and postabsorptive phase may be the major function of circadian oscillators. In the liver of nocturnal rats, the phase of the daily DBP mRNA accumulation profile is severely altered when food is offered exclusively during the day (Ogawa et al., 1997). These findings probably have profound implications on the design of animal studies and possibly even for cell culture experiments.

It is believed that the suprachiasmatic nucleus clock entrains the phase of peripheral clocks via chemical cues, such as rhythmically secreted hormones. It could be demonstrated that temporal feeding restriction under light/dark or dark/dark conditions can change the phase of circadian gene expression in peripheral cell types by up to 12 h while leaving the phase of cyclic gene expression in the suprachiasmatic nucleus unaffected. There-

fore, changes in metabolism can lead to an uncoupling of peripheral oscillators from the central pacemaker. Sudden, large changes in feeding time, similar to abrupt changes in the photoperiod, reset the phase of rhythmic gene expression gradually and are thus likely to act through a clock-dependent mechanism. Food-induced phase resetting proceeds faster in the liver than in the kidney, heart, or pancreas, but after 1 week of daytime feeding, the phases of circadian gene expression are similar in all examined peripheral tissues (Damiola et al., 2000).

J. Influence of Glucocorticoids on Hepatic Circadian Gene Expression

It was shown that dexamethasone induced circadian gene expression in cultured rat-1 fibroblasts and transiently changed the phase of circadian gene expression in liver, kidney, and heart. However, dexamethasone does not affect cyclic gene expression in neurons of the suprachiasmatic nucleus. In contrast to the central clock, circadian oscillators in peripheral tissues appear to remain responsive to phase-resetting throughout the day (Balsalobre et al., 2000).

K. Rat CYP2C6 Expression in Development Is Positively Regulated by D Site-Binding Protein

The CYP2C6 gene becomes maximally transcriptionally activated in livers of postpubertal rats. It was demonstrated in transient transfection experiments with heterologous chloramphenicol acetyltransferase gene constructs and vectors containing cDNAs encoding HNF-1 α , C/EBP- α , and DBP that only DBP was able to activate the CYP2C6 promoter in HepG2 cells. Promoter deletion experiments revealed that the region –38 to –103 nucleotides upstream of the start site of transcription was involved in DBP-mediated activation. A partially purified preparation of DBP produced a footprint between –43 and –64 bp upstream of the transcription start site. With gel mobility shift/Western immunoblot analysis, it was demonstrated that the –40/–65 sequence bound to DBP only in liver nuclear extracts from rats older than 3 weeks; maximal binding was observed by 7 weeks of age, and no binding was detected in liver extracts from rats as young as 1 week old (Yano et al., 1992).

L. Circadian Expression of Rat CYP7 Is Positively Regulated by D Site-Binding Protein

In the rat, hepatic cytochrome P450 cholesterol 7 α -hydroxylase, CYP7, is regulated in vivo at the protein and the mRNA level in response to multiple physiological factors, including liver cholesterol synthesis, bile acid feedback inhibition, and diurnal rhythm. CYP7 is the rate-limiting enzyme in the conversion of cholesterol into bile acids (Lavery and Schibler, 1993; Lee et al., 1994b; Antes et al., 2000; for review, see Cohen, 1999). As with DBP, CYP7 mRNA reaches peak levels in the

evening, and its cycling is independent of daily food and light cues. As predicted for a DBP target gene, the primary level of regulation for CYP7 circadian expression is at the transcriptional level (Lavery and Schibler, 1993; for review, see Cohen, 1999).

DNase I footprinting analysis using bacterially expressed DBP and a cloned 5'-flanking DNA segment of the rat CYP7 gene revealed five distinct DBP-binding sites, designated A through E, distributed between nucleotides (nts) -41 and -295 relative to the CYP7 transcription start site (Lee et al., 1994b). CYP7-directed gene transcription in HepG2 cells transfected with a 5'-CYP7 promoter-chloramphenicol acetyl-transferase reporter was activated up to 12-fold upon cotransfection of a DBP expression vector, whereas an HNF-1 α expression vector did not stimulate CYP7 gene activity. 5'-Deletion analyses and site-specific mutagenesis revealed that this stimulating effect of DBP can in part be ascribed to its functional interaction with DBP-binding sites B (nts -115, -125), C (nts -172, -195), and D (nts -214, -230) (Lee et al., 1994b). In nuclear extracts prepared by a method that, in contrast to conventional techniques, yields near-quantitative recovery of DBP and other nonhistone proteins, the DNA site required for DBP activation is the predominant site of occupancy by nuclear factors on the rat CYP7 promoter. At this site, the predominant binding activity is an evening-specific complex, of which DBP is a component (Lavery and Schibler, 1993). Taken together, the above-mentioned data suggest that DBP may play an important role in cholesterol homeostasis through circadian transcriptional regulation of CYP7 in the rat (Lavery and Schibler, 1993; Lee et al., 1994b).

Alternatively to DBP, the C/EBP- β isoform LAP also bound to the DBP-binding sites, designated A through E in DNase I footprint assays, but effected a more modest increase in rat CYP7-directed gene transcription (up to 3- to 4-fold) when expressed in HepG2 cells (Lee et al., 1994b). Competition for CYP7 promoter-binding sites between the C/EBP- β isoform LAP and the diurnally regulated DBP may be assumed to determine the relative rates of basal versus diurnally regulated CYP7 gene transcription and thus may be a primary mechanism for setting the 3- to 6-fold amplitude that characterizes the circadian rhythm of hepatic CYP7 expression. Since DBP is first expressed in the rat liver 3 to 4 weeks after birth, these findings may account for both the enhanced expression and the onset of the diurnal pattern of CYP7 enzyme levels at this stage of rat development.

IV. Toxicogenomics as an Emerging Science in Toxicology

Acute bromobenzene hepatotoxicity was investigated in rats with a combined toxicogenomics approach integrating the expression analysis of thousands of genes and proteins with classical methods in toxicology (Heijne

et al., 2003). Effects at the molecular level were related to pathophysiological changes of the organism, aiming at a detailed comparison of mechanisms and early detection and prediction of toxicity. Depleted glutathione levels and reduced average body weights were observed 24 h after dosage. These physiological symptoms coincided with many changes of hepatic mRNA and protein content. Only a modest overlap in results from proteomics and transcriptomics was found, and post-transcriptional modifications were not studied. Gene induction experiments confirmed involvement of glutathione *S*-transferase isozymes and epoxide hydrolase in bromobenzene metabolism and identified many genes possibly relevant in bromobenzene toxicity. Observed glutathione depletion coincided with induction of the key enzyme in glutathione biosynthesis, γ -glutamylcysteine synthetase. Oxidative stress was apparent from strong up-regulation of heme oxygenase, peroxiredoxin 1, and other genes. Bromobenzene-induced protein degradation was suggested from two-dimensional gel electrophoresis, up-regulated mRNA levels for proteasome subunits, and lysosomal cathepsin L, whereas genes were also up-regulated with a role in protein synthesis. Both protein and gene expression profiles from treated rats were clearly distinct from controls, as shown by principal component analysis. Several proteins found to be significantly changed upon bromobenzene treatment were identified by mass spectrometry (Heijne et al., 2003).

The largest and most chemically diverse family of nongenotoxic hepatocarcinogens is the peroxisome proliferators (PPs), including hypolipidemic fibrate drugs, plasticizers used in clingwrap/medical tubing, and certain pesticides and solvents. PPs mediate their biological responses via activation of the transcription factor PPAR- α , a member of the nuclear hormone receptor superfamily. PPAR- α activation is responsible for the pleiotropic effects of PPs in rodent liver such as the induction of enzymes of the *b*-oxidation pathway, hepatocyte DNA synthesis, liver enlargement, and tumorigenesis (Chevalier et al., 2000). Fibrate class hypolipidemic drugs such as ciprofibrate activate the PPAR- α . The effects of ciprofibrate (50 mg/kg b.wt. per day for 60 days) on liver gene expression in rats using cDNA microarrays were examined. Ciprofibrate changed the expression of many genes, including previously known PPAR- α agonist-responsive genes involved in processes such as lipid metabolism and inflammatory responses. In addition, many novel candidate genes involved in sugar metabolism, transcription, signal transduction, cell proliferation, and stress responses appeared to be differentially regulated in ciprofibrate-dosed rats. Ciprofibrate also resulted in significant increases in liver weight and hepatocyte proliferation. The cDNA microarray results were confirmed by Northern blot analysis for selected genes (Yadatie et al., 2003). Analysis of the transcript profiles identified changes in the expression

of many genes within several mechanistic pathways that support existing hypotheses regarding peroxisome proliferator-mediated carcinogenicity (Kramer et al., 2003).

Although caution is advisable in the interpretation of genome-wide expression data, the genes identified in the above-mentioned studies provide candidate genes for further studies that may afford new insight into mechanisms of action. In the future it will be possible to discuss the changes in the liver proteome with the background of our knowledge on hierarchical networks involved in pre- and post-transcriptional regulation and post-transcriptional modification of proteins. Therefore, genomic platforms enable the study of network effects in disease and in toxication and holds promise for the development of targeted pharmacological interventions.

V. Conclusion

Considerable evidence stems from knockout and transgenic animal models to demonstrate a pivotal role of C/EBPs and DBP in liver biology and key metabolic functions. For example, C/EBP- α knockout mice die shortly after birth due to hypoglycemia. The published evidence points to a complex network of liver-enriched transcription factors with other regulatory proteins to orchestrate the timely and coordinated expression of liver-specific genes, including the proteins necessary for carbohydrate, lipid, and protein metabolism. Other important features include regulatory functions in cell cycle control and cellular differentiation. The proposed interplay between CDP and C/EBP- α will be the subject of intense research to determine its role in liver tumor biology and particularly the development of hepatocellular carcinoma. Knowledge derived from these studies holds promise for an identification of molecular targets needed for an early diagnosis and/or therapy for malignancy. The importance of C/EBPs in epithelial tumor biology needs to be investigated further to develop a unifying understanding of their role in cellular dedifferentiation and malignant transformation. Rescue of high expression levels of C/EBPs may be an important strategy for molecular therapy of liver tumors based on hepatocellular redifferentiation, as observed during liver regeneration.

A fascinating role of DBP lies in the control of circadian liver gene expression, which seems to be adapted to and entrained by feeding times. It will therefore be of considerable interest to understand the role of DBP in disease. For instance, rhythmic DBP expression is likely to be altered in unconscious intensive care patients with parenteral feeding over prolonged periods of time. The clinically observed deterioration in liver function may be a consequence of desynchronized DBP expression leading to derailed metabolic competence. Likewise, the frequently observed differences in liver gene expression in vivo and in vitro may be the result of disturbed DBP function.

Proteomics and transcriptomics data on protein changes in human livers in pathologic conditions such as viral hepatitis (e.g., hepatitis B or C), primary sclerosing cholangitis, or primary biliary cirrhosis are still missing. In the future, proteome analyses using human tumor tissues, which reflect the pathological state of hepatocellular carcinoma or cholangiocellular carcinoma more closely, will be undertaken. This work will complement the gene expression studies of hepatocellular carcinoma, which are already underway. Efforts have also been directed at the proteome analysis of hepatic stellate cells, since these cells play an important role in liver fibrosis. Since liver fibrosis is reversible but cirrhosis is not, it is of considerable importance to identify therapeutic targets that can slow its progression (Seow et al., 2001). Current limitations of the toxicogenomics approach involving speed of throughput are being overcome by increasing automation and the development of new techniques. The isotope-coded affinity tag method appears particularly promising (Kennedy, 2002). Although new associations between proteins and toxicopathological effects are expected to be uncovered by this new technology, limitations include the fact that this methodology offers a new descriptive dimension without any mechanistic insights and an implied temptation to overemphasize epiphenomena.

Finally, knowledge on mutations and nucleotide polymorphisms of liver-enriched transcription factors will be invaluable for an understanding of diseases. In particular, mutations in the single-exon gene C/EBP- α might impair growth control and cellular differentiation and thus could play an overwhelming role in liver tumors as observed in the differentiation block during acute myeloid leukemia.

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