EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 34, No. 5, 379-384, November 2002

C/EBP binding activity to site F of the rat GLUT2 glucose transporter gene promoter is attenuated by c-Jun *in vitro*

Jae-Woo Kim[1] and Yong-Ho Ahn[1,2]

[1]Department of Biochemistry and Molecular Biology
Institute of Genetic Science
Yonsei University College of Medicine
134 Shinchon-dong, Seodaemoon-gu
Seoul 120-752, Korea
[2]Corresponding author: Tel, 82-2-361-5187;
Fax, 82-2-312-5041; E-mail: yha111@yumc.yonsei.ac.kr

Accepted 4 November 2002

Abbreviations: C/EBP, CCAAT/enhancer binding protein; GLUT2, glucose transporter type 2; HNF, hepatocyte nuclear factor; EMSA, electrophoretic mobility shift assay

Abstract

The expression of the GLUT2 glucose transporter gene in liver is suppressed in cultured hepatoma cell lines and primary cultured hepatocytes. Earlier report showed that CCAAT/enhancer binding protein (C/EBP) regulates the promoter activity of the rat GLUT2 glucose transporter gene in liver cells. C/EBPα and C/EBPβ activated the promoter activity by binding to at least two regions of the promoter and one of the C/EBP binding sites, named as site F, also has the AP-1 binding consensus. In this study, we investigated whether the AP-1 can influence on C/EBP binding to this site. The addition of recombinant c-Jun protein with liver extract caused the attenuation of C/EBP binding to site F with the appearance of a new shifted band. The shifted band was competed out with the addition of unlabeled AP-1 consensus oligonucleotide, indicating that c-Jun also can bind to site F. Another C/EBP site on GLUT2 promoter, site H, did not bind AP-1. Analysis of the DNA-protein complex revealed that C/EBP and c-Jun bind to site F in mutually exclusive manner rather than form heterodimeric complex with each other. From these results, it is suggested that the transcriptional activation of C/EBP may be influenced by c-Jun protein in certain status of the liver cells, such as acute phase response, as well as hepatocarcinogenesis.

Keywords: C/EBP, c-Jun, DNA-protein binding, GLUT2 promoter, hepatocyte

Introduction

The GLUT2, a specific facilitative glucose transporter, is expressed mainly in liver cells and pancreatic β-cells, and has a higher K_M value compared to other types (Gould *et al.*, 1991). The glucose metabolism in liver begins or ends with the movement of glucose into or out of the hepatocyte through this type of glucose transporter (Craik and Elliott, 1979). The GLUT2, however, fails to be transcribed in established hepatoma cells such as HepG2 (Mueckler *et al.*, 1985; Thorens *et al.*, 1988) or in culture of primary hepatocytes, where the expressions of other liver-specific genes are maintained (Clayton and Darnell, 1983; Bucher *et al.*, 1990; Mischoulon *et al.*, 1992). This phenomenon suggests the possibility that the GLUT2 gene may be regulated not only in a tissue specific manner, but also by growth-differentiation status of liver cells. Therefore, studies on the gene regulation of GLUT2 may provide an insight into understanding the molecular mechanism related to the liver-specific gene expression.

Previously, we reported that CCAAT/enhancer binding protein (C/EBP) regulated the promoter activity of the rat GLUT2 gene in liver cells (Kim and Ahn, 1998). C/EBP is one of the major liver-enriched *trans*-acting factors that play key roles in the transcriptional regulation of genes in liver cegIls (Johnson *et al.*, 1987; Landschulz *et al.*, 1988; Descombes *et al.*, 1990). C/EBP and C/EBP activated the promoter activity by binding to at least two regions of the promoter, site F and site H. In addition, the binding of C/EBP was markedly decreased when liver cells were subjected to primary culture, suggesting that C/EBP might be important for the maintenance of normal liver glucose metabolism.

The proteins of the Jun family are transcription factors involved in several basic cellular activities that govern differentiation and proliferation and in several signal transduction pathways (Gutman and Wasylyk, 1991; Karin, 1992). These proteins, as well as C/EBP, all belong to the class of the basic leucine zipper proteins that bind DNA as dimer. In addition, c-Jun was found to participate in the modulation of certain liver-specific regulatory elements, such as the phosphoenolpyruvate carboxykinase promoter (Gurney *et al.*, 1992). It may also participate in the general mech-

anism that allows correct development of the liver (Hilberg *et al.*, 1993) or modulation of liver gene expression during liver regeneration (Hsu *et al.*, 1992).

In this study, we found that one of the C/EBP sites "site F" on the GLUT2 promoter also can bind c-*jun* product *in vitro*. The binding of C/EBP in normal liver cells was decreased gradually as higher amounts of c-Jun were added to the binding reaction, suggesting that c-Jun competes out C/EBP binding, rather than heterodimerizes with C/EBP. Supershift assay showed clearly shifted c-Jun-DNA complexes with a combination of rat liver nuclear extract. It was considered that c-Jun might compete the physiological binding of C/EBP in certain status such as acute phase response, as well as hepatocarcinogenesis.

Materials and Methods

General methods

Standard procedures were carried out by the methods described by Sambrook *et al.* (1989). The oligonucleotides used in this study are as follows: site F, 5'-GCCTCTACTCTTATCTGACTCAACAGG-3'; site H, 5'-AGTTAACAATCTTGATTTCCACATCACAAACGTGCA-3'; C/EBP, 5'-ATTCAATTGGGCAATCAGGAATT-3' (Landschulz *et al.*, 1988). AP-1 consensus oligonucleotide was purchased from Promega. All synthesized oligonucleotides were purified from polyacrylamide gel.

Preparation of nuclear extracts

Nuclear extracts from liver of male Sprague-Dawley rats were prepared as described by Gorski *et al.* (1986). Protein concentration was determined according to Bradford (1976). The extracts were frozen in aliquots and stored at -70°C. Purified recombinant c-Jun protein was purchased from Promega.

Electrophoretic mobility shift assay (EMSA) and supershift assay

DNA-protein binding reactions were performed using 50,000 cpm of probe per reaction in a solution containing 10 mM HEPES, pH 7.9, 60 mM KCl, 7% glycerol, 1 mM EDTA, 1 mM DTT, 2 μg of poly (dI-dC), and the indicated amount of nuclear extracts. After 30-min on ice, the samples were resolved in 4% non-denaturing polyacrylamide gel polymerized in 0.25×TBE. For competition assay, 100-200-fold molar excess of oligonucleotides for specific transcription factors were added to the binding reactions prior to the addition of nuclear extracts. For C/EBP supershift assay, 1 μg of anti-C/ EBPα or anti-C/EBPβ (SantaCruz Biotech) was added to the DNA-protein binding reaction. Also, for AP-1 supershift assay we used following antibodies; anti-c-Jun/AP-1 (D), broadly reactive with Jun family proteins; anti-c-Jun/AP-1 (N), c-Jun-specific; anti-c-Fos (Craik and Elliott, 1979; Clayton and Darnell, 1983; Mueckler *et al.*, 1985; Thorens *et al.*, 1988; Bucher *et al.*, 1990; Gould *et al.*, 1991; Mischoulon *et al.*, 1992), broadly reactive with Fos family proteins; anti-c-Fos (Mischoulon *et al.*, 1992), c-Fos specific. The dried gels were exposed to X-ray film at -70°C with an intensifying screen.

Results and Discussion

We earlier reported the protein-binding sites of the rat GLUT2 gene promoter and showed that C/EBP regulates the promoter activity of the rat GLUT2 glucose transporter gene in liver cells. C/EBPα and C/EBPβ activated the promoter activity by binding to at least two regions of the promoter (Figure 1). Although the protein complex bound to site F in normal liver extract did not contain AP-1 (Kim and Ahn, 1998), we found that the sequence of site F has matched with AP-1 consensus. This observation prompted us to investigate whether the addition of AP-1 protein to the reaction could influence the C/ EBP binding. As shown in Figure 2, site F binds C/EBPα and C/EBPβ, which are normally expressed in liver. When recombinant human c-Jun protein was added with liver extract, a new strong protein-DNA complex appeared; suggesting that c-Jun protein also could bind to site F. Because the normal liver nuclear extract contains only little amount of c-Jun protein, it was considered that EMSA of site F with normal liver extract did not show to bind AP-1 protein in the previous report (Kim and Ahn, 1998). Meanwhile, the addition of c-Jun protein alone in the reaction failed to show DNA-protein complex as shown in Figure 2, suggesting that the DNA-protein complex is not Jun/ Jun homodimer, but heterodimer between Jun and

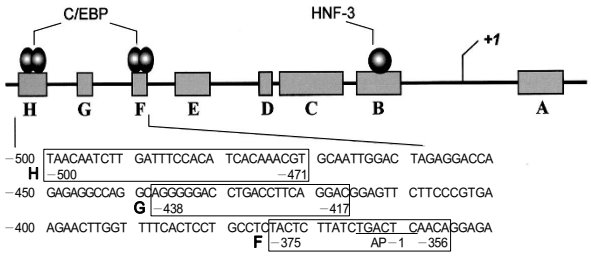

Figure 1. Structure of the rat GLUT2 promoter. Transcription initiation site is assigned +1 (Ahn *et al.*, 1995) and the boxed areas represent the protected regions of rat GLUT2 promoter identified by DNase I footprinting assay using rat liver nuclear extract (Kim and Ahn, 1998). Site H and F were binding sites for C/EBP, and site B was for HNF-3. The nucleotide sequences focused in this study were shown below.

other protein that might exist in normal liver. Jun protein belongs to the class of the basic leucine zipper proteins, as C/EBP does, and it is well known that proteins of this class can form heterodimer each other. Therefore, we next investigated whether C/EBP and Jun protein bound to site F simultaneously form-

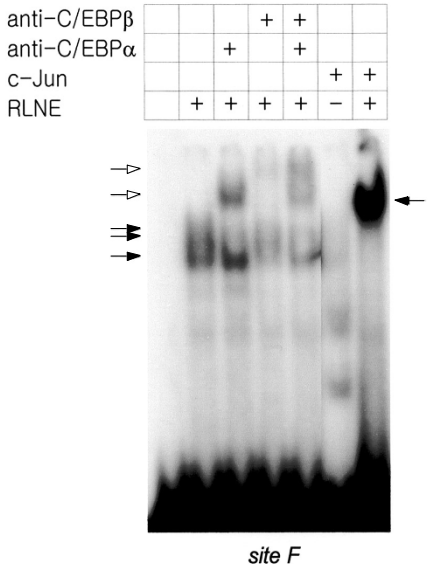

Figure 2. Electrophoretic mobility shift assay (EMSA) of site F. EMSA using site F oligonucleotide as a probe with rat liver nuclear extract (RLNE, 10 μg of protein per reaction) and/or c-Jun protein was carried out in the absence or presence of anti-C/EBPα, and/or anti-C/EBPβ antibody as indicated above, and the resulting complexes were electrophoresed in 4% non-denaturing gel. The DNA- protein complexes are indicated by filled arrows; the positions of supershifted DNA-protein-antibody complexes are indicated by open arrows.

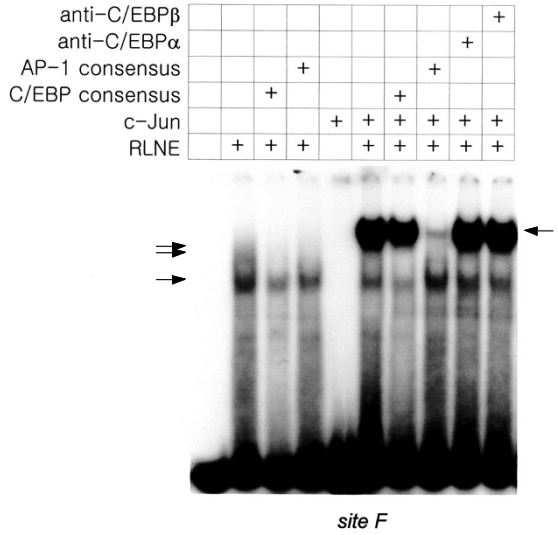

Figure 3. Competition and supershift assay of AP-1 band of site F. The labeled site F oligonudeotide was incubated with rat liver nuclear extract (RLNE, 10 μg) and/or c-Jun protein in the absence or presence of 100-fold molar excess of competing oligonucleotides for known transcription factors, as indicated at the top of each lane. Also, anti-C/EBPα, and/or anti-C/EBPβ antibodies were added as indicated. The resulting complexes were electrophoresed in 4% non- denaturing gel. The DNA-protein complexes are indicated by filled arrows.

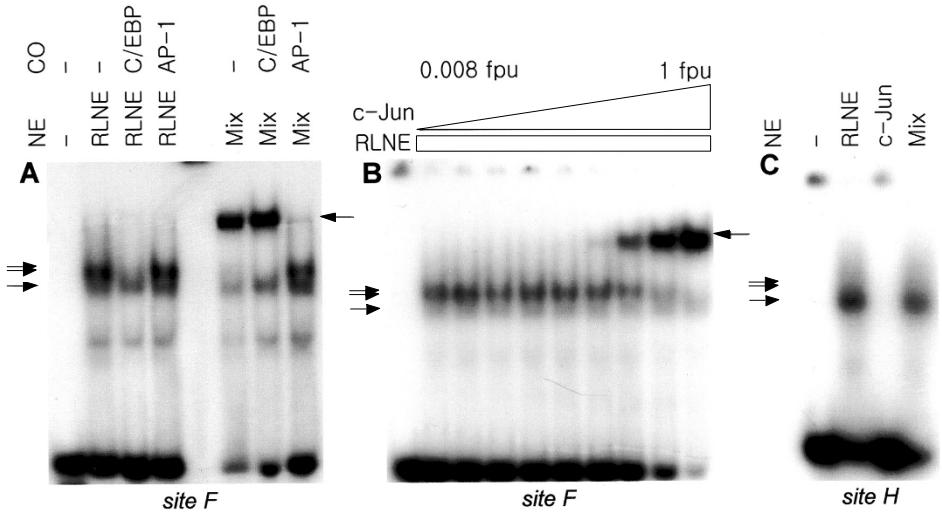

Figure 4. C/EBP and c-Jun binds to site F in mutually exclusive fashion on EMSA. The labeled site F oligonucleotide was incubated with rat liver nuclear extract (RLNE, 10 μg) and/or c-Jun protein. A. EMSA with competitors. RLNE alone or mixed with c-Jun protein was incubated with site F probe in the absence or presence of 100-fold molar excess of competing C/EBP or AP-1 consensus oligonucleotides as indicated at the top of each lane. B. Titration of C/EBP by addition of c-Jun protein. The equal amount of RLNE and increasing amount of c-Jun protein was incubated with the labeled site F probe. C. EMSA using site H oligonucleotide, which has another C/EBP binding site but not AP-1 consensus site. The resulting complexes were electrophoresed in 4% non-denaturing gel. The DNA-protein complexes are indicated by filled arrows.

ing heterodimer. As shown in Figure 3, the protein-DNA complex disappeared with the addition of AP-1 consensus as a competitor, while the C/EBP consensus failed to compete out. Also, anti-C/EBPα or anti-C/EBPβ did not bind to the shifted complex, suggesting that the proteins bound to site F could not be a result from dimerization of C/EBP and c-Jun protein.

With tight control in the amounts of these proteins, we found that the intensity of the Jun protein band increased when unlabeled C/EBP consensus competitor was added to the reaction (Figure 4A). This suggests that the site F is binding Jun protein, but a small portion of site F is still binding C/EBP protein. Also, the addition of AP-1 competitor led to the reappearance of the C/EBP band, suggesting that these two proteins bind to site F in mutually exclusive fashion. This result was further confirmed by the EMSA with the gradual increase of c-Jun protein, which directly showed Jun protein competed out the C/EBP binding (Figure 4B). In other words, it is thought that GLUT2 promoter is occupied by C/EBP in normal physiological situation, but when the cell is changed by certain external stimuli that result in abnormal expression of Jun protein, GLUT2 promoter can be occupied by Jun instead of C/EBP. This phenomenon was observed only in site F, while another C/EBP binding site (site H) did not showed any difference when c-Jun was added (Figure 4C). From this result, it might be suggested that the abnormal events like the appearance of the Jun protein, can lead to an abnormal control of liver-specific genes, like GLUT2.

AP-1 transcription factor consists of more than the single protein encoded by c-*jun* or c-*fos*. In fact, there are several additional members of the AP-1, that is, Jun-B and Jun-D in Jun family, and Fos-B, Fra-1, and Fra-2 in Fos family, respectively. It is known that heterodimer formation and cooperative DNA binding includes multiple members of both Jun and Fos families with the exception that Fos alone neither forms dimers nor binds to DNA (Rana *et al.*, 1995). To investigate whether any of these proteins functions as a partner of Jun protein on site F, we performed supershift assay using various antibodies against Jun/Fos family proteins (Figure 5). Anti-c-Jun/AP-1(D) antibody, which is broadly reactive with highly conserved DNA binding domain of c-Jun, Jun B, and Jun D, completely blocked the DNA-protein complex. Supershifted band appeared when c-Jun-specific antibody was added to the binding reaction as expected. However, antibodies reactive with Fos family failed to affect the binding properties. This suggests the possibility that the Jun protein bound to site F interacts other protein instead of Fos family protein, which remains to be clarified by other experiments.

The fact that C/EBP bind to the GLUT2 promoter provides a significant insight in relation to hepatocyte differentiation. In liver, C/EBPα mRNA has been observed to decrease when hepatocytes are grown in culture or during liver regeneration (Mischoulon *et al.*, 1992), as well as hepatoma-derived cell line such as HepG2 (Friedman *et al.*, 1989). These observation,

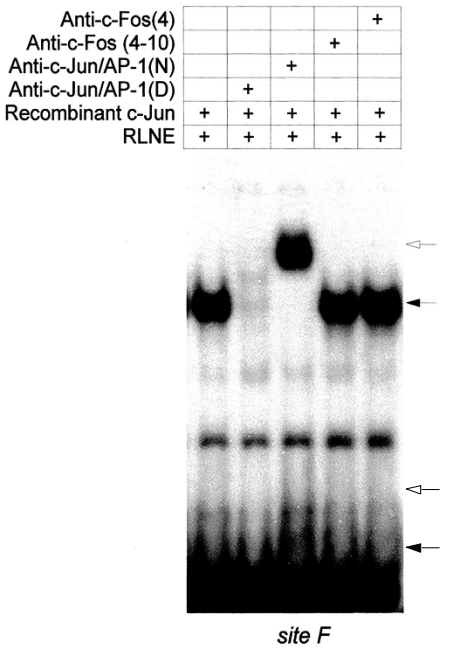

Figure 5. Supershift assay with Jun/Fos family antibodies. Electrophoretic mobility shift assay using site F oligonucleotide as a probe with rat liver nuclear extract (RLNE, 10 μg of protein per reaction) and c-Jun protein was carried out in the absence or presence of indicated antibodies. Anti-c-Jun/AP-1 (D) is broadly reactive with highly conserved DNA binding domain of c-Jun, Jun B, and Jun D, while anti-c-Jun/AP-1 (N) is specific for c-Jun protein. Also, anti-c-Fos (4-10) is broadly reactive with c-Fos, Fos B, Fra-1, and Fra-2, whereas anti-c-Fos (4) is a specific antibody against c-Fos. The resulting complexes were electrophoresed in 4% non-denaturing gel. The DNA- protein complexes are indicated by filled arrows; the positions of supershifted DNA-protein-antibody complexes are indicated by open arrows.

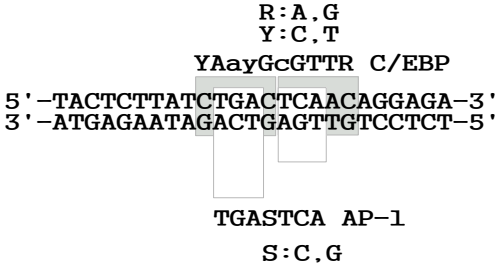

Figure 6. C/EBP and AP-1 binding sites on site F of GLUT2 promoter. The matched nucleotides with consensus are indicated by capital letters. Gray and white boxes show C/EBP and AP-1 molecules bound on this site, respectively.

together with the fact that GLUT2 mRNA was down-regulated during primary hepatocyte culture (Mischoulon *et al*., 1992), suggests a special role of C/EBPα for the appearance of GLUT2 in hepatocyte differentiation program as well as the regulation of the GLUT2 gene transcription in response to physiological stimuli in fully differentiated hepatocytes. On the other hand, several ways of interplay between AP-1 and C/EBP have been observed. It was reported that isolation of hepatocytes by a collagenase perfusion method activated expression of several immediate-early growth response genes including AP-1, resulting in an extensive inhibition of C/EBPα expression (Rana *et al*., 1994). Other report has shown the direct association of AP-1 and C/EBP. Hsu *et al*. (1994) reported that Fos and Jun repress transcription activation by C/EBPβ through association at the basic zipper region in the absence of DNA, by altering the binding specificity of C/EBP. As shown in Figure 6, C/EBP and AP-1 binding sites are overlapped in site F of the GLUT2 promoter. In this study, we demonstrated the AP-1 and C/EBP could bind to the same region by direct competition of DNA binding, providing another mechanism by which AP-1 may inhibits the role of C/EBP. The c-*jun* mRNA was reported to be increased during chemical hepatocarcinogenesis (Sakai *et al*., 1989). Meanwhile, Rana *et al*. (1994) showed that C/EBPα and immediate-early gene transcription factors were reciprocally expressed during hepatocyte growth-differentiation program. Our results will provide an insight into the molecular mechanisms involved in the regulation of the GLUT2 expression during the development and differentiation of liver cells.

Acknowledgements

This work was supported by the Yonsei University Research Fund of 2001.

References

Ahn YH, Kim JW, Han GS, Lee BG, Kim YS. Cloning and characterization of rat pancreatic β-cell/liver type glucose transporter gene: a unique exon/intron organization. Arch Biochem Biophys 1995;323:387-96

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-64

Bucher NLR, Robinson GS, Farmer SR. Effects of extracellular matrix on hepatocyte growth and gene expression: implications for hepatic regeneration and the repair of liver injury. Semin Liver Dis 1990;10:11-9

Clayton DF, Darnell JE Jr. Changes in liver-specific compared to common gene transcription during primary culture of mouse hepatocytes. Mol Cell Biol 1983;3:1552-61

Craik JD, Elliott KRF. Kinetics of 3-O-methyl-D-glucose transport in isolated rat hepatocytes. Biochem J 1979;182:503-8

Descombes P, Chojkier M, Lichtsteiner S, Falvey E, Schibler U. LAP, a novel member of the C/EBP gene family, encodes a liver enriched transcriptional activator protein. Genes Dev 1990;4:1541-51

Friedman AD, Landschulz WH, McKnight SL. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes Dev 1989; 3:1314-22

Gorski K, Carneiro M, Schibler U. Tissue-specific *in vitro* transcription from the mouse albumin promoter. Cell 1986; 47:767-76

Gould GW, Thomas HM, Jess TJ, Bell GI. Expression of human glucose transporters in Xenopus oocytes: kinetic characterization and substrate specificities of the erythrocyte (GLUT1), liver (GLUT2) and brain (GLUT3) isoforms. Biochemistry 1991;30:5139-45

Gurney AL, Park EA, Giralt M, Liu J, Hanson RW. Opposing actions of Fos and Jun on transcription of the phosphoenolpyruvate carboxykinase (GTP) gene. Dominant negative regulation by Fos. J Biol Chem 1992;267:18133-9

Gutman A, Wasylyk B. Nuclear targets for transcription regulation by oncogenes. Trends Genet 1991;7:49-54

Hilberg F, Aguzzi A, Howells N, Wagner EF. c-jun is essential for normal mouse development and hepatogenesis. Nature 1993;365:179-81.

Hsu JC, Bravo R, Taub R. Interactions among LRF-1, JunB, c-Jun, and c-Fos define a regulatory program in the G1 phase of liver regeneration. Mol Cell Biol 1992;12:4654-65.

Johnson PF, Landschulz WH, Graves BJ, McKnight SL. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev 1987;1:133-46

Karin M. Signal transduction from cell surface to nucleus in development and disease. FASEB J 1992;6:2581-90

Kim JW, Ahn YH. CCAAT/enhancer binding protein regulates the promoter activity of the rat GLUT2 glucose transporter gene in liver cells. Biochem J 1998;336:83-90

Landschulz W, Johnson PF, Adashi EY, Graves BJ, McKnight SL. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev 1988;2:786-800

Mischoulon D, Rana B, Bucher NLR, Farmer SR. Growth-dependent inhibition of CCAAT enhancer-binding protein (C/EBPα) gene expression during hepatocyte proliferation in the regenerating liver and in culture. Mol Cell Biol 1992;12: 2553-60

Mischoulon D, Rana B, Kotliar N, Pilch PF, Bucher NLR, Farmer SR. Differential regulation of glucose transporter 1 and 2 mRNA expression by epidermal growth factor and transforming growth factor-beta in rat hepatocyte. J Cell Physiol 1992;153:288-96

Mueckler M, Caruso C, Baldwin SA, Panico M, Blench I, Morris HR, Allard WJ, Lienhard GE, Lodish HF. Sequence

and structure of a human glucose transporter. Science 1985; 229:941-5

Rana B, Mischoulon D, Xie Y, Bucher NLR, Farmer SR. Cell-extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes: Reciprocal expression of C/EBPα and immediate-early growth response transcription factors. Mol Cell Biol 1994;14:5858-69

Rana B, Xie Y, Mischoulon D, Bucher NLR, Farmer SR. The DNA binding activity of C/EBP transcription factors is regulated in the G1 phase of the hepatocyte cell cycle. J Biol Chem 1995;270:18123-32

Sakai M, Okuda A, Hatayama I, Sato K, Nishi S, Muramatsu M. Structure and expression of the rat *c-jun* messenger RNA: Tissue distribution and increase during chemical hepatocarcinogenesis. Cancer Res 1989;49:5633-7

Sambrook J, Fritsch EF, Maniatis T, Molecular cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory, NY, 1989

Thorens B, Sarkar HK, Kaback HR, Lodish HF. Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney, and β-pancreatic islet cells. Cell 1988;55:281-90