INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®
ROLE OF SPECIFIC CCAAT/ENHANCER-BINDING PROTEIN ISOFORMS IN
INTESTINAL EPITHELIAL CELLS

By

Ionela GHEORGHIU

Département d'Anatomie et de Biologie Cellulaire
Faculté de Médecine
Université de Sherbrooke

In partial fulfillment of the requirements of the Degree of Master of Science

Sherbrooke, Québec, August 2001
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-74388-8
3.1 Nuclear extracts .................................................................................................... 20
3.2 Preparation of $^{32}$P-labelled oligonucleotides .................................................. 21
3.3 Electrophoretic mobility shift assay ..................................................................... 21
4. Protein extraction and Western analysis ............................................................... 22
5. RNA extraction and Northern analysis .................................................................... 23
  5.1 RNA extraction .................................................................................................... 23
  5.2 Northern blot ...................................................................................................... 24
  5.3 Preparation of radioactive probes ....................................................................... 24
  5.4 Prehybridation and hybridation ......................................................................... 25
6. Differential display .................................................................................................... 26
  6.1 mRNA purification ............................................................................................... 27
  6.2 Reverse transcription of mRNA .......................................................................... 27
  6.3 PCR reactions ...................................................................................................... 29
  6.4 Electrophoresis .................................................................................................... 30
  6.5 Analysis of differential bands .............................................................................. 31
7. Doubling time calculation ......................................................................................... 32
  7.1 Doubling time ..................................................................................................... 32
  7.2 Statistical analysis ............................................................................................... 33
8. DNA laddering .......................................................................................................... 33

RESULTS ......................................................................................................................... 35

1. Effect of C/EBP isoform expression on the regulation of acute phase proteins and
C/EBPs ........................................................................................................................... 35
  1.1 Effect of C/EBP isoform expression on C/EBP endogenous protein levels ...... 35
1.2 Effect of C/EBP isoform expression on the regulation of acute phase proteins and C/EBPs .............................................................. 36

2. Effect of C/EBP isoform expression on cell growth and anoikis ................. 38
  2.1 Effect of C/EBP isoform expression on cell growth ................................ 38
  2.2 Effect of C/EBP isoform expression on anoikis .................................... 39
  2.3 Identification of differentially expressed genes ....................................... 39

DISCUSSION .................................................................................................. 53
  1. C/EBP isoforms are involved in the regulation of the acute phase protein gene haptoglobin ........................................................................................................ 53

2. C/EBP isoforms are not involved in the regulation of α-acid glycoprotein in intestinal epithelial cells .............................................................. 54

3. C/EBP isoforms are not subject to autoregulation in IEC-6 cells ............... 55

4. C/EBPβ LAP overexpression leads to a G1/S delay of intestinal epithelial cells ..... 57

5. C/EBPβ LAP overexpression seems to protect IEC-6 cells from apoptosis .... 58

6. Identification of differentially expressed genes .......................................... 59

CONCLUSIONS AND PERSPECTIVES ...................................................... 61

ACKNOWLEDGEMENTS .............................................................................. 64

REFERENCES ............................................................................................... 65
LIST OF FIGURES

Figure 1. Sequential model of induction for acute phase genes................................. 4
Figure 2. The structure of C/EBP isoforms α, β and δ............................................. 9
Figure 3. The cell cycle.......................................................................................... 16
Figure 4. Coding regions of C/EBPα p30, C/EBPβ LAP and LIP, C/EBPδ, 3hF and 4hF introduced in the retroviral vector pBabepuro .................................................. 41
Figure 5. Composition of C/EBP DNA-binding complexes in IEC-6 cell lines
   overexpressing C/EBP isoforms ...................................................................... 42
Figure 6. C/EBPβ endogenous protein levels are increased in C/EBPα p30 and C/EBPδ
   overexpressing cell clones ............................................................................. 43
Figure 7. Pattern of expression of haptoglobin, α-acid glycoprotein and C/EBP isoforms in C/EBP overexpressing clones .................................................. 44
Figure 8. Composition of C/EBP DNA-binding complexes in IEC-6 cell lines
   overexpressing dominant-negative proteins ............................................... 45
Figure 9. Pattern of expression of haptoglobin, α-acid glycoprotein and C/EBP isoforms in dominant-negative overexpressing clones ........................................... 46
Figure 10. Time course of pRb phosphorylation and p42/p44 MAP kinase activation in control and C/EBPβ LAP overexpressing clones ........................................... 48
Figure 11. Time course of cyclin D1/2, cylin E and p27kip1 expression in control and C/EBPβ LAP overexpressing clones .................................................. 49
Figure 12. DNA laddering of different C/EBP isoforms expressing clones ............. 50
Figure 13. Analysis of differentially expressed genes ............................................. 51
LIST OF TABLES

Table 1. C/EBP genes and phenotypic characterization of knockout models .................. 12
Table 2. Growth characteristics of IEC-6 cells overexpressing C/EBP isoforms .......... 47
Table 3. Gene similarities when compared to genes from the Genbank Database .......... 69
LIST OF ABBREVIATIONS

AGP: α-acid glycoprotein

API: activator protein-1

APP: acute phase protein

APR: acute phase response

ATP: adenosine triphosphate

bZIP: basic region and leucine zipper including domain

C/ATF: C/EBP-related activating transcription factor

CDK: cyclin-dependent kinases

C/EBP: CCAAT/enhancer-binding protein

CHOP: C/EBP homologous protein

CKI: cyclin kinase inhibitors

CO₂: carbon dioxide

cps: counts per second

CREB: cAMP response element-binding protein

C-terminal: carboxy-terminal

dCTP: deoxycytidine-5′-triphosphate

DD: differential display

DEPC: diethyl pyrocarbonate

DMEM: Dulbecco's modified Eagle medium

DNA: deoxyribonucleic acid
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
FBS: fetal bovine serum
IEC-6: rat intestinal epithelial cells
IGF I: insulin-like factor I
IGF II: insulin-like factor II
IkB: NF-κB inhibitor
IL: interleukin
IL-R: interleukin receptor
IFN-γ: interferon-γ
IPTG: isopropyl-β-thiogalactopyranoside
LB: L-broth
LIP: liver-enriched transcriptional inhibitor protein
LAP: liver enriched transcriptional activator protein
LZ: leucine zipper
MAP kinase: mitogen activated protein kinase
MOPS: sulphonic acid 3-(N-morpholino propane) sodium
NF-κB: nuclear factor kappa B
NP-40: nonidet P-40
N-terminal: amino-terminal
OD: optical density
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PEPCK: phosphoenolpyruvate carboxykinase

PMSF: phenylmethylsulfonyl fluoride

pRb: retinoblastoma protein

RT-PCR: reverse transcription-polymerase chain reaction

SDS: sodium dodecyl sulfate

STAT: signal transducer and activator of transcription

TBE: tris borate buffer

TBS: tris buffered saline

TE: tris EDTA buffer

TEMED: tetramethylethlenediamine

TGFβ: transforming growth factor β

TNF-α: tumor necrosis factor α

TNFR: TNF-α receptor

TRIS: tris (hydroxymethyl) aminomethane
LIST OF PUBLICATIONS


COMMUNICATIONS


désacétylases dans la lignée épithéliale intestinale IEC-6. Association canadienne-française pour l'avancement des sciences, Montréal, PQ, Canada.


Role of specific CCAAT/enhancer-binding protein isoforms in intestinal epithelial cells

By Ionela GHEORGHIU

In partial fulfillment of the requirements of the Degree of Master of Science
Département d'Anatomie et de Biologie Cellulaire, Faculté de Médecine,
Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4.

ABSTRACT

Intestinal epithelial cells participate in the acute phase response (APR) during intestinal inflammation. We have shown that acute phase protein (APP) genes are induced during the intestinal APR, and that the CCAAT/enhancer binding protein family of transcription factors are involved in APP regulation. To determine the role of different C/EBP isoforms in vivo, we generated IEC-6 rat intestinal epithelial cell lines stably expressing different C/EBP isoforms and dominant-negative C/EBPs, by infection with the retroviral vector pBabepuro. Overexpression of C/EBPα p30 and C/EBPδ led to increases in C/EBPβ LAP and C/EBPβ LIP endogenous protein levels, as determined by electrophoretic mobility shift assays and Western blot. C/EBPα p30, C/EBPβ LAP and
C/EBPδ were directly involved in the glucocorticoid-, cAMP- and IL-1-dependent induction of APP gene haptoglobin. Inhibition of C/EBP activity with dominant-negative C/EBPs (C/EBPβ LIP, 3hF, 4hF) decreased haptoglobin gene responsiveness to these regulators. These data show that the three C/EBPs isoforms are involved in the regulation of haptoglobin. α-acid glycoprotein induction by glucocorticoids was independent of dominant-negative C/EBPs or C/EBP isoform expression. In addition, the response of C/EBPβ and C/EBPδ to various regulators was not affected by the overexpression of different C/EBP isoforms or dominant-negative C/EBPs, in contrast to other cell types. C/EBPβ LAP expressing cells showed an increased doubling time characterized by a delay in pRb inactivation in response to serum, a decrease in cyclin D1/2 and cyclin E proteins levels and by an increase in p27Kip1 protein levels. In addition, C/EBPβ LAP seems to protect intestinal epithelial cells against anoikis. Thus, specific C/EBP isoforms are involved in the differential expression of APP genes in response to hormones and cytokines. Moreover, C/EBP isoforms may play an important role in the control of intestinal epithelial cell growth and apoptosis. Our results confirm the implication of C/EBP isoforms in intestinal inflammation.
Les cellules intestinales épithéliales contribuent à une réponse inflammatoire aiguë (RIA) lors de l'inflammation de l'intestin. Nous avons démontré que la RIA induit l'expression de protéines de la réponse inflammatoire et que les facteurs de transcription C/EBPs sont impliqués dans leur régulation. Afin de déterminer le rôle de différents isoformes de C/EBPs in vivo, la lignée intestinale épithéliale de rat IEC-6 a été infectée avec des vecteurs rétroviraux pBabepuro encodant différents isoformes des C/EBPs et des dominant-négatifs de C/EBPs. Les études de gel de rétention et l'analyse Western montrent que les isoformes C/EBP\(\beta\) LAP et C/EBP\(\beta\) LIP endogènes augmentent dans les cellules avec des niveaux élevés de C/EBPa p30 et C/EBP\(\delta\). C/EBPa p30, C/EBP\(\beta\) LAP et C/EBP\(\delta\) sont directement impliqués dans l'induction de l'haptoglobine en réponse aux glucocorticoïdes, l'IL-1 et la forskoline. L'inhibition de l'activité des C/EBPs avec des dominant-négatifs (C/EBP\(\beta\) LIP, 3hF, 4hF) réduit l'induction du gène haptoglobine par ces régulateurs. Ces résultats démontrent que les trois isoformes C/EBPs sont impliqués dans la régulation de l'haptoglobine. L'induction de l'\(\alpha\)-glycoprotéine acide par les glucocorticoïdes est indépendante des isoformes C/EBPs. De plus, la réponse de C/EBP\(\beta\) et C/EBP\(\delta\) aux différents régulateurs n'est pas affectée par la surexpression des C/EBPs ou des dominant-négatifs, contrairement à d'autres types cellulaires. L'augmentation des niveaux d'expression de C/EBP\(\beta\) LAP cause un retard du cycle cellulaire. Ceci est caractérisé par un retard dans l'inactivation de pRb en réponse au sérum, une diminution des niveaux des cycline D1/2 et cycline E et une augmentation des niveaux de p27\(^{kip1}\). Aussi, C/EBP\(\beta\) LAP semble protéger les cellules intestinales contre l'anoikose. En
conclusion, les isoformes spécifiques des C/EBPs sont impliqués dans l'expression des protéines de la réponse inflammatoire. Les facteurs de transcription C/EBPs semblent aussi être impliqués dans le contrôle de la croissance et de l'apoptose des cellules intestinales épithéliales. Nos résultats confirment l'implication des isoformes des C/EBPs dans l'inflammation de l'intestin.
INTRODUCTION

1. Inflammation and the acute phase response

Injury, trauma or infection of a tissue is followed by a series of host reactions in the effort to prevent ongoing tissue damage, to isolate and destroy the infectious organism and to activate the repair process. This homeostatic process is known as inflammation, and the early and immediate set of reactions induced are known as the acute phase response (APR). The cells most associated with initiating the APR are the macrophages. Activated macrophages release a large spectrum of mediators, including cytokines (Baumann and Gauldie, 1994).

Cytokines are divided in three groups: proinflammatory cytokines (TNFα, IL-1, IL-8, IFN-γ), interleukin-6-type cytokines (IL-6, IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1) and anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGFβ) (Koj and Guzdek, 1995). At the reactive site, systemic IL-1 and TNFα cause the release of a secondary wave of cytokines. This secondary wave increases the homeostatic signal and initiates the cellular and cytokine cascades involved in the APR (Boumann and Gauldie, 1994). The systemic reaction following cytokine release is characterized by fever, leukocytosis, increased release of several hormones, activation of clotting and of the complement cascade and drastic modulations of plasma protein synthesis (Koj, 1996).
Acute phase proteins (APPs) are plasma proteins which play important roles in the control of inflammation. They are produced mainly by the liver, but also by other organs like the intestine (Fiocchi and Podolsky, 1995). The APPs participate in the clearing of harmful agents, in tissue repair, in the inactivation of serine proteases (e.g. elastase and cathepsin G, liberated at the inflammatory sites) (Baumann and Gauldie, 1994; Engler, 1995). For example, the acute phase protein haptoglobin binds the hemoglobin produced following a tissue hemolyse, phenomena which characterize each acute phase response (Engler, 1995). The acute phase proteins can be divided in two classes, according to their response to cytokines (Koj et al., 1993). The set of APP genes regulated by IL-1-type cytokines form the type-1 APPs, while those regulated by IL-6-type cytokines alone or in combination with glucocorticoids, are the type-2 APPs. The stimulation of type-1 APP genes is synergistically enhanced by IL-6-type cytokines and sometimes by glucocorticoids.

There are four major classes of transcription factors involved in the regulation of acute phase proteins: NF-κB, STAT, glucocorticoid receptors and C/EBPs (Baumann and Gauldie, 1994; Mackiewicz, 1997). Poli (1998) proposed for hepatocytes a model of the cascade of events leading to the activation of APPs. In this model, activated STAT3 is involved in the IL-6-induced up-regulation of C/EBPβ and C/EBPδ gene promoters. On the other hand, the binding of TNFα and IL-1β to their membrane receptors induces the phosphorylation and the degradation of IκB, the inhibitor of NF-κB. In this way, NF-κB is liberated and transferred to the nucleus where it participates in the activation of various
APP genes. TNFα and IL-1β also activate the MAP kinase pathway which leads to the phosphorylation and activation of different transcription factors like C/EBPβ, involved in the expression of APP genes (Fig. 1). Induction of both C/EBPβ and C/EBPδ levels by inflammatory stimuli occurs in most tissues analyzed, suggesting a more general role of these two factors in inflammation (Akira et al., 1990; Kinoshita et al., 1992). Indeed, functional C/EBP-binding motifs (initially known as type-1 IL-6-responsive elements (IL-6 REs)) have been characterized on the promoters of most type-1 APP genes (haptoglobin, hemopexin, α1-acid glycoprotein, serum amyloid A1, A2 and A3, complement C3, C-reactive protein), often in association with IL-1-responsive NF-κB (Poli, 1998).
Figure 1. Sequential model of induction for acute phase genes.
2. The intestinal acute phase response

Intestinal epithelial cells form a critical mucosal barrier between the host's internal milieu and the external environment, and function as an integral component of the mucosal immune system (Chang et al., 1995). The inflammatory response in the gastrointestinal tract is mediated by the concerted action of cellular and humoral elements, including cytokines and reactive oxygen metabolites (Mayer, 2000). In addition, bacterial products are also responsible for the induction of intestinal inflammation (Mayer, 2000). An increasing body of evidence has demonstrated that intestinal epithelial cells can actively participate in the generation of an immune response (Sanderson and Walker, 2000). The first pro-inflammatory cytokine discovered to be synthesized by the intestinal epithelial cells was IL-6 (Shirota et al., 1990). Following this, other pro-inflammatory cytokines (IL-1, IL-6, IFN-γ) were found to be produced by intestinal epithelial cells during inflammatory response (Sartor, 1995). Finally, intestinal epithelial cells present receptors for different cytokines. Stadnyk and Waterhouse (1997) have shown that intestinal epithelial cells produce and respond to IL-1, both in vitro and in vivo. Thus, intestinal epithelial cells participate in intestinal homeostasis and mucosal immunity by secreting and responding to cytokines (Fiocchi and Podolsky, 1995).

The intestinal inflammatory response leads to the establishment of an acute phase response characterized by the local production of acute phase proteins (APPs) in response to cytokines and hormones (Podolski and Fiocchi, 2000). For example, results obtained in our laboratory have demonstrated that the APP gene haptoglobin is induced in various rat
models of intestinal inflammation and in the rat intestinal epithelial cell line IEC-6 in response to cAMP and glucocorticoids. We have also shown that C/EBPα, C/EBPβ and C/EBPδ were expressed and modulated in intestinal epithelial cells in vivo and in vitro (Blais et al., 1995; Boudreau et al., 1996, 1997, 1998; Yu et al., 1999). Other results show that C/EBPβ and C/EBPδ isoforms are involved in the regulation of APP gene haptoglobin by cAMP (Pelletier et al., 1998) and TGFβ (Yu et al., 1999) in intestinal epithelial cells. It is known that the prolongation of inflammatory process could lead to different chronic diseases, like ulcerative colitis and Crohn's disease at intestinal level (Sartor, 1994; Fiocchi, 1997, 1998). Still, many events regarding the initiation and propagation of intestinal acute phase response remain to be clarified.

3. The C/EBP family of transcription factors

The CCAAT/enhancer binding protein family of transcription factors is a class of transactivators involved in the regulation of acute phase protein expression in various tissues, including the intestine (Poli, 1998). The prototypic C/EBP is a modular protein consisting of an activation domain, a DNA-binding basic region and a leucine-rich dimerization domain. The dimerization domain, known as "leucine zipper", is an amphipathic α-helix with a hydrophobic surface containing repeated leucine residues at intervals of seven amino acids (Landschulz et al., 1988, 1989). The basic region and the leucine zipper form together the "b-zip" motif (Cao et al., 1991; Williams et al., 1991; Johnson, 1993) also found in other transcription factors (AP1, CREB, C/ATF).
The first member of the C/EBP family, designated C/EBPα, was originally purified from rat liver nuclear extracts as a heat-stable DNA-binding protein recognizing viral enhancer core sequences (Johnson et al., 1987). Five other isoforms were later discovered: C/EBPβ (Cao et al., 1991), C/EBPδ (Cao et al., 1991), C/EBPγ (Roman et al., 1990), C/EBPε (Williams et al., 1991) and C/EBPξ or CHOP (Ron and Habener, 1992). The C/EBP isoforms bind DNA as homodimers or heterodimers between the members of the same family (Landschulz et al., 1989; Descombes et al., 1990) or between a member of the C/EBP family of transcription factors and another transcription factor like NF-κB (LeClair et al., 1992; Ray et al., 1995; Vietor et al., 1996) or Fos/Jun (Vinson et al., 1993).

The N-terminal portion of C/EBPα contains three separate domains that cooperatively activate transcription (Nerlov and Ziff, 1995) (Fig. 2). C/EBPβ also contains three N-terminal activation domains (Kowenz-Leutz et al., 1994). In addition, both C/EBPα and C/EBPβ bear negative regulatory or inhibitory domains, which mask the activity of the transactivation domains (Pei and Shih, 1991) and of the DNA-binding b-zip domain (Williams et al., 1995). Phosphorylation of C/EBPα and C/EBPβ in the inhibitory domains results in the liberation of the transactivation and DNA-binding domains from such inhibitory interactions (Kowenz-Leutz et al., 1994). Another interesting feature of these two isoforms is that two different proteins can be generated from the same gene. This is made possible by a leaky ribosomal scanning mechanism. The protein p30 retains the dimerization and DNA-binding domains, but possesses an altered transactivation potential compared with the long form of C/EBPα, p42 (Lin et al., 1993; Ossipow et al., 1995).
1993). Like C/EBPα, two C/EBPβ isoforms are generated: a full-length 32 kDa protein, LAP (liver activator protein), and a truncated protein of 20 kDa, LIP (liver inhibitory protein), which lacks the transactivation domains and acts as a dominant-negative (Williams et al., 1995). Regarding C/EBPδ, less is known about its structure. Some recent work done in our laboratory has shown the presence of transactivation domains between amino acid 30 and 100, and of negative regulatory domains between amino acid 100 and 158. C/EBPδ presents certain homologies with C/EBPα and C/EBPβ. For example, the negative regulatory elements of C/EBPδ are located in the C-terminus of the protein, close to the basic region, organization similar to that of C/EBPβ. Moreover, the transactivation domains of C/EBPδ are located in the N-terminal region, like those of C/EBPα and C/EBPβ (Fig. 2). In the following sections I will focus on the C/EBP isoforms α, β and δ. These three isoforms are involved in acute phase response and are the object of my research.
Figure 2. The structure of C/EBP isoforms α, β and δ.

The numbers correspond to the amino acid number. BR: basic region; LZ: leucine zipper; dark squares: positive element; grey squares: negative element.
4. Role of C/EBPs in different tissues

The C/EBP family of transcription factors is involved in the regulation of various aspects of cellular differentiation and function in multiple tissues. C/EBPα plays a key role in adipocyte differentiation by activating genes that encode proteins involved in creating the adipocyte phenotype (Darlington et al., 1998). It is also involved in cell cycle arrest because C/EBPα both activates the transcription and induces the post-transcriptional stabilization of p21, an inhibitor of cyclin-dependent kinases (Timchenko et al., 1996). C/EBPα regulates terminal hepatocyte differentiation and function, and controls the expression of hepatic enzymes involved in energy metabolism (insulin-responsive glucose transporter, PEPCK) and of factors that maintain liver-specific functions (transferrin, factor IX, IGF I, IGF II) (Lee et al., 1997). C/EBPα is also involved in the control of the APR in liver (Burgess-Beusse and Darlington, 1998). In addition to a role in the early processes of adipocyte differentiation, C/EBPβ and C/EBPδ are direct mediators of the APR in hepatocytes (Poli, 1998) and in intestinal epithelial cells (Boudreau et al., 1997; Yu et al., 1999; Désilets et al., 2000). Furthermore, C/EBPβ is involved in the control of cell proliferation in liver (Diehl, 1998) and is essential for normal development and function of the mammary gland (Robinson et al., 1998). Along with its role as a mediator of the APR in hepatocytes and intestinal epithelial cells, C/EBPδ is involved in the regulation of proliferation and differentiation of adipocytes (Darlington et al., 1998) and hepatocytes (Diehl, 1998), in the differentiation of lung epithelial cells (Breed et al., 1997) and myelomonocytic cells (Scott et al., 1992), in
mammary epithelial cells G0 growth arrest and programmed cell death (O'Rourke et al., 1999).

Transient transfection studies have shown that C/EBP isoforms control the levels of C/EBPβ (Chang et al., 1995) and C/EBPδ (Yamada et al., 1998) mRNAs by autoregulatory mechanisms in many cell types. Furthermore, the murine C/EBPα promoter presents a C/EBP consensus site that allows autoregulation by direct binding of C/EBPα as well as activation by other C/EBP family members (Christy et al., 1991). An autoregulatory mechanism for C/EBPα has also been described for human cells (Timchenko et al., 1995). Thus, C/EBP isoforms play a role in controlling their own expression.

The important roles determined for the members of C/EBP family in tissue culture models have been proven by developing knockout mice for different C/EBP isoforms. For example, animals lacking C/EBPα fail to accumulate lipids, to activate gluconeogenic pathways at birth and present defects in glycogen storage. The mutant homozygotes die 7-8 hours postpartum because of severe hypoglycemia (Flodby et al., 1996; Wang et al., 1995). C/EBPβ(−/−)δ(−/−) mice have extremely high rates of mortality. These mice display reduced adipocyte differentiation and fail to accumulate lipid droplets in brown adipose tissue (Tanaka et al., 1997) (Table1).
Table 1. C/EBP genes and phenotypic characterization of knockout models.
<table>
<thead>
<tr>
<th>Name</th>
<th>Alternative name</th>
<th>Expression patterns</th>
<th>Phenotypic abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBPα</td>
<td>C/EBP</td>
<td>Liver, adipose, intestine, lung, adrenal gland, placenta, ovary, PBMC</td>
<td>Hepatocyte proliferation, perinatal lethal, Defective lipid storage, defective carbohydrate metabolism, Myeloid maturation blocked at myeloblast stage</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>NF-IL-6, IL-6DBP</td>
<td>Liver, intestine, lung, adipose</td>
<td>None detected, Defective carbohydrate metab., defective lipid storage (synergistic with C/EBPδ), Immunodeficient, defective Th1 response, Macrophage phagosome defect</td>
</tr>
<tr>
<td></td>
<td>LAP, CRP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGP/EBP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NF-M, ApC/EBP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/EBPγ</td>
<td>Ig/EBP</td>
<td>Ubiquitous</td>
<td>None detected, Defective lipid storage (synergistic with C/EBPδ), None detected</td>
</tr>
<tr>
<td>C/EBPδ</td>
<td>CELF.CRP3</td>
<td>Liver, lung, adipose, intestine</td>
<td>None detected</td>
</tr>
<tr>
<td></td>
<td>NF-IL6b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rec/EBP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/EBPε</td>
<td>CRP1</td>
<td>Myeloid and lymphoid lineages</td>
<td>None detected, None detected</td>
</tr>
<tr>
<td>C/EBPζ</td>
<td>CHOP/Gadd 153</td>
<td>Ubiquitous</td>
<td>None detected</td>
</tr>
</tbody>
</table>
5. C/EBPs and the cell cycle

The cell cycle describes the process leading to cellular division. It contains five phases: G0, G1, S, G2 and M. In the G0 phase, the cells present a weak metabolic activity (quiescence). Following a stimulation for growth, the cells enter the cell cycle. During the G1 phase, the cells grow and synthesize the proteins needed to enter S phase, which corresponds to DNA replication. S phase is followed by G2 phase, when the cells are prepared for mitosis. Finally, during the M phase, the cells divide (Carosella and Leteurtre, 1995).

The progression through the cell cycle is controlled by specific kinases, known as cyclin dependent kinases (CDK). Their activity is regulated by the association with the cyclins, which are regulatory proteins specific for each phase of the cell cycle. The activity of CDK complexes is controlled by phosphorylation and binding of cyclin kinase inhibitors (CKI) (Hunter and Pines, 1994; Morgan, 1995). The CDKs, the catalytic subunits responsible for the progression of the cell cycle, are serine/threonine kinases of 33-35 kDa. The cyclins show variable concentrations during the cell cycle and contain a 100-amino acid conserved domain responsible for CDK binding and activation (Hunter and Pines, 1994; Morgan, 1995). Seven CDKs have been identified until now: CDK1 (also known as Cdc2) associates with cyclin A and B and is active during the G2 phase; CDK2 associates with cyclin A and is active during the S phase. The activity of CDK2 to CDK6 is specific to the G1 phase and these CDKs associate with specific G1 phase cyclins. The cyclins C, D1, D2, D3 and E are specific for the G1 phase. The cyclins A and F are
involved in the S and the G2 phases, while the cyclins B1 and B2 are responsible for the propagation through the G2 phase and G2/M transition (Hunter and Pines, 1994; Morgan, 1995). The cyclin kinase inhibitors bind to CDK-cyclin complexes and inhibit their kinase activity. They act in three ways: some bind CDKs and inhibit their action and others form a complex with cyclin-CDK. Finally, some are phosphatases and inactivate the CDKs (Hunter and Pines, 1994; Morgan, 1995). The most important CKIs are p21 which induces cell cycle arrest in the G1 phase following DNA damage. p27Kip1 which lead to a G1 phase arrest after TGFβ or cAMP treatment. p15 which is induced by TGFβ. p16 and p24 (Darbon et al., 1995).

The cell cycle presents a series of checkpoints at which the cell will stop and assess intra- and extracellular conditions before going on to the next stage (Hartwell, 1978). These checkpoints are located at the G1/S phase or G2/M transitions and at the beginning of the M phase. The transition from the G1 to the S phase is under the control of the pRb (retinoblastoma) protein. Rb is a family of three proteins: p107, p110 and p130. p110Rb intervene in G1 phase. In its hypophosphorylated form, pRb binds the transcription factor E2F that controls the expression of proteins required for the progression through the S phase. Upon phosphorylation of pRb by cyclin-dependent kinases, pRb loses its affinity for the transactivation domain of E2F and this domain is released (Helin et al., 1993; Mittnacht, 1998). E2F is then able to transactivate promoters of genes encoding proteins like α-polymerase, dihydrofolate reductase (DHFR), etc. (Fry et al., 1999; Hsiao et al., 1994; Means et al., 1992) (Fig. 3).
A link between C/EBPα and the cell cycle has been recently demonstrated. Indeed, C/EBPα is expressed in highly differentiated nondividing cells such as hepatocytes, adipocytes and a subset of cells in the lung (Birkenmeier et al., 1989). Studies in recent years have shown that C/EBPα is involved in cell growth arrest. This depends in part on the transcriptional regulation of the cyclin-dependent kinase inhibitor p21 (Timchenko et al., 1996) or its stabilization at the protein level (Timchenko et al., 1997) in different cell lines. More recently, C/EBPα has been shown to inhibit cell growth by direct repression of E2F-mediated transcription (Slomiany et al., 2000) and may disrupt E2F-p107 complexes (Timchenko et al., 1999). It has been shown that all C/EBP isoforms bind to E2F4, by their C-terminal region, but only C/EBPα p42 (Hendrick-Taylor and Darlington, 1995; Slomiany et al., 2000) and C/EBPβ LAP (Gheorghiu et al., 2001) induce growth arrest. We are the first to show that there is an interaction between C/EBPβ LAP and E2F4 transcription factor, leading to reduced expression of E2F target genes and decreased proliferation.
Figure 3. The cell cycle.

The presence of different factors involved in the regulation of cell cycle is indicated. CDK: cyclin-dependent kinase; CKI: cyclin-kinase inhibitor; Rb: retinoblastoma protein.
Cyclins B/A + Cdc2

Cyclin A + CDK2

Cyclin E + CDK2

Cyclin D + CDK4/6

Rb dephosphorylation

Mitogens

CKIs (p16, p21, p27)
6. Research project

6.1 Effect of C/EBP isoforms on the regulation of acute phase proteins and C/EBPs

As mentioned before, C/EBP isoforms are involved in many important biological processes like adipocyte and hepatocyte differentiation, mediation of acute phase response in hepatocyte, differentiation of lung epithelial cells and myelomonocytic cells etc. However, their exact role in the control of intestinal APP gene expression or intestinal epithelial cell proliferation is not known. To address this issue we generated by retroviral infection IEC-6 intestinal epithelial cell lines and populations stably expressing different C/EBP isoforms and dominant-negative C/EBPs. Then, using those cell lines and populations, we have shown that the three C/EBP isoforms are involved in the regulation of haptoglobin. In addition, we found that C/EBPβ, C/EBPδ and α-glycoprotein expression are not regulated by C/EBP isoforms in contrast to other cell types.

6.2 Effect of C/EBP isoform expression on cell cycle and anoikis

We have observed that cell lines that overexpress C/EBPβ LAP were growing slower than cell lines expressing the other C/EBP isoforms. We therefore verified the expression of cyclin D1/2, cyclin E and p27Kip1 and the phosphorylation status of pRb and p42/p44 MAP kinase. By Western blot analysis, we demonstrated that pRb inactivation and hyperphosphorylation in response to serum was delayed in C/EBPβ LAP clones, as compared to control cells. Furthermore, cyclin D1/2 and cyclin E expression was decreased, while p42/p44 were normally induced in response to serum. We also observed
an increase in p27\textsuperscript{Kip1} expression levels. We also identified differentially expressed genes by comparing C/EBP\textbeta LIP (the truncated form of C/EBP\textbeta which functions as a dominant-negative) expressing cells with C/EBP\textbeta LAP expressing cells, by using the differential display method. Finally, we observed that cells expressing C/EBP\textbeta LAP have reduced DNA fragmentation after 24 hours in suspension, suggesting that overexpression of C/EBP\textbeta LAP may protect against anoikis. This results demonstrate that C/EBP isoforms may be involved in cell cycle progression and anoikis in intestinal epithelial cells.
MATERIAL AND METHODS

1. Cell culture

The rat intestinal epithelial cell line IEC-6 was provided by A. Quaroni (Cornell University, Ithaca, NY). Cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL, Burlington, ON), containing 5% fetal bovine serum (FBS) (BioMédia, Drummondville, QC), 2 mM of L-glutamine (Gibco-BRL, Burlington, ON) and 100 U/ml penicillin and streptomycin (Gibco-BRL, Burlington, ON) at 37°C in an atmosphere containing 95% air and 5% CO₂. Cells at 80% confluence were stimulated for 24 hours with or without forskolin (1 μM) (Calbiochem, La Jolla, CA), human recombinant IL-1β (10 ng/ml) (R & D Systems, Minneapolis, MN) or 10⁻⁶M dexamethasone (Sigma-Aldrich Canada, Oakville, ON). To determine the status of pRb and p42/p44 MAP kinase phosphorylation and cyclin D, cyclin E and p27kip1 expression, IEC-6 cells were growth-arrested for 48 hours in DMEM without serum before stimulation with DMEM containing 5% FBS.

2. Retroviral constructs and infection

The coding sequences for C/EBPα p42 and p30 (Lin et al., 1993), C/EBPβ LAP and the dominant-negative transrepressor C/EBPβ LIP (Yeh et al., 1995), and C/EBPδ (Cao et al., 1991) were cloned in the EcoRI site of the retroviral vector pBabepuro (Morgenstern and Land, 1990). The DNA-binding C/EBP dominant-negative 3hF and 4hF sequences
(Krylov et al., 1995; Moitra et al., 1998) were PCR-amplified and subcloned in the same retroviral vector. 293T cells (human kidney cells transformed by T antigen) (gift from Alain Nepveu, McGill University) were used for the transfections. Once the cells in 100 mm dishes reached confluence, they were transferred to 60-mm dishes. The next day, the medium was changed with 2.5 ml DMEM containing 10% FBS and the cells were transfected using Lipofectamine 2000 (Gibco-BRL, Burlington, ON) (1 µl Lipofectamine 2000/50 µl Opti-MEM), according to manufacturer's instructions. Two days after transfection, supernatants were filtered and stored at -80°C. IEC-6 cells at 40% confluence in 60 mm dishes were infected for 24 hours with 2 ml of virus-containing supernatant in the presence of 8 µg/ml Polybrene (Sigma-Aldrich Canada, Oakville, ON). The next day the medium was changed. After 24 hours, the cells were split in selection medium containing 2 µg/ml puromycin (Sigma-Aldrich Canada, Oakville, ON). Empty vector, C/EBPα p30, C/EBPβ LAP, C/EBPβ LIP and C/EBPδ IEC-6 infected clones, and 3hF or 4hF cell populations were selected after three weeks.

3. Electrophoretic mobility shift assays

3.1 Nuclear extracts

The cells were treated for 24 hours with different agents and the nuclear extracts were prepared according to Stein et al. (1989). The cells were washed two times with 3 ml of PBS 1X, harvested in 1 ml of PBS 1X and centrifuged 2 minutes at 1500 RPM. Then, the cells were resuspended in 100 µl lysis buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate pH 7.9, 1 mM ethylenediaminetetraacetic acid (EDTA), 60
mM KCl, 0.5% NP-40. 1 mM phenylmethylsulfonyl fluoride (PMSF). lysed 5 minutes on ice and centrifuged 30 seconds at 13000 RPM. The pellet containing the nuclei was re-suspended in 100 µl of resuspension buffer (NRB) (0.25 M Tris-HCl pH 7.8. 60 mM KCl, 1 mM DTT, 1.5 mM PMSF), followed by three cycles of 2 minutes of freezing in liquid nitrogen and thawing at 37°C. The samples were then centrifuged at 4°C for 10 minutes at 13000 RPM. The supernatant was recuperated, frozen quickly in liquid nitrogen and stored at -80°C.

3.2 Preparation of 32P-labelled oligonucleotides

The -166 to -145 hapto A C/EBP DNA-binding site from the rat haptoglobin promoter (5'-CCAAGTATGAAGCAAGAGCTCA-3') (Pajovic et al., 1994) was labelled with 32P-dCTP and the Klenow fragment of DNA polymerase I from E.coli (Amersham Pharmacia Biotech, Baie d'Urfé. QC).

3.3 Electrophoretic mobility shift assay

Nuclear extracts (10 µg) were incubated for 30 minutes at room temperature in a 20 µl volume containing 2 µl reaction buffer 10X (10 mM Tris-HCl pH 7.5. 500 mM NaCl. 10 mM EDTA. 50% glycerol). 0.25 µg dIdC (Amersham Pharmacia Biotech. Baie d'Urfé. QC) and 1 µl of fresh radioactive probe of 50 cps (count per second) intensity. The samples were separated on a 4% polyacrylamide gel containing 0.5X TBE buffer (45 mM Tris-borate, 40 mM boric acid, 1 mM EDTA) and 2% glycerol, during 2 hours at 150 volts after a pre-electrophoresis of 30 minutes at 150 volts. The gel was dried at 80°C for 1 hour, then exposed at -80°C in a cassette with a BioMax MR film (Kodak. Rochester.
Supershift assays were performed by adding 1 µl of rabbit affinity-purified polyclonal antibody raised against C/EBP isoforms (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes before the addition of the radioactive probe.

4. Protein extraction and Western analysis

Nuclei from different cell lines were solubilized in sample buffer (62.5 mM Tris-HCl, pH 6.9, 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 10% glycerol and 0.04% bromophenol blue) and sonicated on ice. To determine the status of pRb and p42/p44 MAP kinase phosphorylation, total cellular extracts were isolated from empty or C/EBPβ LAP vector infected IEC-6 cell clones treated without serum or after 10 minutes, 4, 16 and 24 hours of induction with serum. To determine the expression of cyclin D1/2, cyclin E and p27Kip1, empty or C/EBPβ LAP expressing cells were treated without or with serum for 2, 4, 8, 16 and 24 hours and total cellular extracts isolated. The cells were washed two times with 3 ml of PBS 1X. Then, the cells were resuspended in 250 µl sample buffer. After 10 minutes at room temperature, the lysed cells were transferred in 1.5 ml tubes, heated for 5 minutes at 100°C and sonicated on ice. Protein concentrations were measured by the Bradford method (Bio-Rad Protein assay kit, Bio-Rad Laboratories, Mississauga, ON). Proteins (50 µg) were resolved on a SDS gel containing 10% polyacrylamide (SDS-PAGE) as described by Laemmli (1970). The proteins were transferred to a PVDF membrane (Roche Molecular Biochemicals, Laval, Qc) for 2 hours at 4°C and 50 volts, in a transfer buffer (0.025 M Tris-Cl pH 7.6, 0.192 mM glycine, 10% methanol). Then, the membrane was blocked for 1 hour at room temperature in a
blocking solution: TBS 1X (20 mM Tris-Cl pH 7.6, 137 mM NaCl) containing 5% non-fat dry milk and 0.1% Tween-20. Incubation with affinity-purified rabbit polyclonal antibodies (1/1000) specific to C/EBP isoforms (Santa Cruz Biotechnology, Santa Cruz, CA) was performed at 4°C overnight. Incubations with the mouse monoclonal antibody against pRb (14001A) (PharMingen, Mississauga, ON), with phospho p42/p44 MAP kinase (Thr 202/Tyr 204) antibody (New England Biolabs, Mississauga, ON) and with the rabbit polyclonal antibody against cyclin D1/2, cyclin E and p27Kip1 (Santa Cruz Biotechnology, Santa Cruz, CA) were also performed at 4°C overnight. After three washes of 10 minutes with PBS 1X-Tween-20 0.1%, the membrane was incubated with the secondary antibody (1/1000) (Sigma-Aldrich Canada, Oakville, ON) in a blocking solution for 1 hour at room temperature. After three washes with PBS 1X-Tween-20 0.1%, the immune complexes were detected with the Super Signal West Pico Substrate system (Pierce, Rockford, IL) following the manufacturer's instructions. The membrane was exposed in an autoradiography cassette with a Hyperfilm ECL (Amersham Pharmacia Biotech, Baie d'Urfé, QC). Protein loading was verified by Coomassie blue staining.

5. RNA extraction and Northern analysis

5.1 RNA extraction

After induction for 24 hours with different agents, total cellular RNA was isolated by the guanidinium isothiocyanate-phenol extraction method (Chomczynski and Sacchi. 1987).
using the TRlzol reagent (Gibco-BRL, Burlington, ON), according to the manufacturer's instructions.

5.2 Northern blot

Northern analysis was performed using the method described by Fourney et al. (1988). Total RNA (20 - 40 µg) were denatured for 15 minutes at 65°C and then separated by 1% agarose gel electrophoresis for 5 hours at 70 volts. The agarose gel contains MOPS buffer (10 mM sulfonic acid 3-(N-morpholino propane) sodium, 50 mM EDTA, 48 mM sodium acetate) and 5% formaldehyde. Then, the RNAs were transferred to a Nylon membrane (Nytan, Schleicher and Schuell, Keene, NH) by diffusion (Sambrook et al., 1989) for 16 hours in 10X SSC solution (1.5 M sodium chloride, 150 mM tri-sodium citrate). The membrane was then dried for 2 hours at 80°C and exposed to UV for 2 minutes in a DNA fixation apparatus (Fotodyne, BioCan Scientific, Mississauga, ON).

5.3 Preparation of radioactive probes

The haptoglobin and α-acid glycoprotein fragments were isolated by RT-PCR from rat liver mRNAs (Pelletier et al., 1998; Boudreau et al., 1998). The C/EBPβ and C/EBPδ fragments were provided by Steve McKnight (Cao et al., 1991). The murine α-tubulin fragment was provided by René St-Arnaud (Hôpital Shriner's, Montréal). The plasmids were digested for 2 hours at 37°C with different restriction enzymes (Amersham Pharmacia Biotech, Baie d'Urfé, QC). The fragments were then isolated on a 0.8% agarose gel and purified. The band containing the fragment was cut out of the gel and placed in a bottom pierced tube that contained mineral wool. This tube was placed on
another tube and centrifuged for 10 minutes at 13000 RPM. The solution collected in the tube was then purified by extraction with phenol-chloroform-isoamyl alcohol (25: 24: 1). The DNA was precipitated with 1/10 volume of sodium acetate 3 M and two volumes of 100% cold ethanol. After a 5 minutes centrifugation, the DNA was resuspended in water. The fragments were labelled with α-32P-dCTP, using a Multiprime DNA labelling system (Amersham Pharmacia Biotech, Baie d'Urfé, QC).

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Length</th>
<th>Restriction site</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haptoglobin</td>
<td>545 bp</td>
<td>HindIII, BamHI</td>
<td>Pelletier et al. (1998)</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>1.5 kb</td>
<td>EcoRI, Xhol</td>
<td>Cao et al. (1991)</td>
</tr>
<tr>
<td>C/EBPδ</td>
<td>1.0 kb</td>
<td>EcoRI, BamHI</td>
<td>Cao et al. (1991)</td>
</tr>
<tr>
<td>AGP</td>
<td>144 bp</td>
<td>Pvu II</td>
<td>Boudreau et al. (1998)</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>1.6 kb</td>
<td>Pst I</td>
<td>Lemischka et al. (1981)</td>
</tr>
</tbody>
</table>

**5.4 Prehybridation and hybridation**

Nylon membranes with fixed RNAs were incubated for 4 hours at 65°C in a prehybridation solution (0.12 M Tris-HCl pH 7.4, 0.6 M NaCl, 8 mM EDTA, 0.1% sodium pyrophosphate, 0.1% SDS, 0.06% heparin) and then overnight at 65°C with the radioactive probe in a hybridation solution (prehybridation solution + 10% dextran sulphate and 0.625 mg/ml heparin). The next day, the membrane was washed for 30 minutes in a 0.1 X SSC and 0.1% SDS solution at 65°C. The membrane was exposed at -80°C in a cassette with a BioMax MR film (Kodak, Rochester, NY).
Differential display is a method that allows the identification and isolation of differentially expressed genes.

**Differential Display**

mRNA Population

| CAAAAAAAAAA-An | GAAAAAAAAAAA-An | UAAAAAAAAAAA-An |

I. Reverse Transcription

5' -AAGCTTTTTTTTTTTG-3' (H-T11G)
dNTPs
MMLV reverse transcriptase

| CAAAAAAAAAA-An | GTTTTTTTTTTTTCGAA |

II. PCR Amplification

5' -AAGCTTGGATTGCC-3' (H-AP 1)
5' -AAGCTTTTTTTTTTTTTTGG-3' (H-T11G)
dNTPs
α-[³²P-dATP]
Taq DNA polymerase

| AAGCTTGGATTGCC | GTTTTTTTTTTTTCGAA |

| AAGCTTGGATTGCC | GTTTTTTTTTTTTCGAA |

III. Denaturing Polyacrylamide gel

RNA Sample: X Y

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Negative electrode (-)

Positive electrode (+)
6.1 mRNA purification

Total RNAs from C/EBPβ LAP and LIP IEC-6 infected cells were obtained as described in section 5.1. Poly A+ RNAs were isolated with the Oligotex procedure (Qiagen, Mississauga, ON). An amount of 250 µg total RNA was mixed with DEPC (diethyl pyrocarbonate – inhibitor of RNAases) treated water in a total volume of 250 µl. Then, 250 µl of 2 X binding buffer (20 mM Tris-HCl pH 7.5, 1000 mM NaCl, 2 mM EDTA and 0.2% SDS) and 15 µl of Oligotex suspension were added. The tubes were incubated for 3 minutes at 65°C and 10 minutes at room temperature. After 2 minutes centrifugation at 13000 RPM, the supernatant was removed from the tubes and the Oligotex resin containing the mRNA, was resuspended in 400 µl of wash buffer OW2 (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA). The contents of the tubes were transferred to spin columns and centrifuged for 30 seconds at 13000 RPM. The spin columns were then transferred to another microcentrifuge tubes and 400 µl of wash buffer were applied to them and centrifuged for 30 seconds at 13000 RPM. Finally, the mRNA was eluted with 20 µl of preheated (70°C) elution buffer (5 mM Tris-HCl pH 7.5).

6.2 Reverse transcription of mRNA

The Core RT mix G (with anchored G primer) was prepared as follows (RT core mix with other anchored primers was prepared in a similar way):
4 tubes were labelled for RT using anchored G primer as follows:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>RNA sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1G</td>
<td>Number 1</td>
</tr>
<tr>
<td>2G</td>
<td>Number 2</td>
</tr>
<tr>
<td>3G</td>
<td>Number 3</td>
</tr>
<tr>
<td>4G</td>
<td>Number 4</td>
</tr>
</tbody>
</table>

17 µl of the RT core mix G were added to each of the four tubes containing 2 µl each of freshly diluted RNA samples (0.1 µg/µl). The RT reaction was carried out on a thermocycler following the kit protocol:

65°C, 5 min → 37°C, 60 min → 75°C, 5 min → 4°C soak.
6.3 PCR reactions

PCR tubes were labelled as follows:

<table>
<thead>
<tr>
<th>1G1</th>
<th>2G1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1G2</td>
<td>2G2</td>
</tr>
<tr>
<td>1G3</td>
<td>2G3</td>
</tr>
<tr>
<td>1G4</td>
<td>2G4</td>
</tr>
<tr>
<td>1G5</td>
<td>2G5</td>
</tr>
<tr>
<td>1G6</td>
<td>2G6</td>
</tr>
<tr>
<td>1G7</td>
<td>2G7</td>
</tr>
<tr>
<td>1G8</td>
<td>2G8</td>
</tr>
</tbody>
</table>

The first number stands for the RNA sample (1 – C/EBPβ LIP, 2 – C/EBPβ LAP), the second letter denotes the nature of the anchored primer, and the third number stands for the arbitrary primer. 2 µl of the corresponding H-AP primer were added to each tube at room temperature (ex: H-AP1 to 1G1, 2G1; H-AP2 to 1G2, 2G2 etc.). The 36X PCR core mix was prepared as follows:

<table>
<thead>
<tr>
<th></th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>360.0</td>
</tr>
<tr>
<td>10 PCR reaction buffer</td>
<td>72.0</td>
</tr>
<tr>
<td>dNTP (25 µM)</td>
<td>57.6</td>
</tr>
<tr>
<td>HT₁₁G primer (2 µM)</td>
<td>72.0</td>
</tr>
<tr>
<td>Taq Polymerase (5 U/ml) (Qiagen)</td>
<td>7.2</td>
</tr>
<tr>
<td>α-[³³P] dATP (200 Ci/mmole) (NEN)</td>
<td>7.2</td>
</tr>
<tr>
<td>Total</td>
<td>576.0</td>
</tr>
</tbody>
</table>
4 tubes were labelled as follows:

RT1G
RT2G
RT3G
RT4G

144 µl of Core Mix G were added to each of the above tubes. Then, 18 µl of the corresponding RT reaction were also added to the above tubes (18 µl of RT tube 1G goes into RT1G, 18 µl of RT tube 2G goes into RT2G, etc.). Finally, 18 µl of RT1G were added to each of 1G1, 1G2, 1G3, 1G4, 1G5, 1G6, 1G7 and 1G8 and 18 µl of RT2G were added to each of 2G1, 2G2, 2G3, 2G4, 2G5, 2G6, 2G7 and 2G8. In the end, 30 µl of mineral oil were added to each tube. The PCR reactions were carried out on a thermocycler following the kit protocol:

40 cycles of: 94°C, 30 min → 40°C, 2 min → 72°C, 30 sec →
1 cycle of: 72°C, 5 min → 4°C soak.

6.4 Electrophoresis

3.5 µl of each sample were mixed with 1.5 µl of loading buffer and loaded onto a non-denaturing 6% acrylamide gel. The electrophoresis was performed in 0.5X TBE buffer (45 mM Tris-borate, 40 mM boric acid, 1 mM EDTA). The gel was transferred to filter paper, dried under vacuum and exposed at -80°C in a cassette with a X-Omat RP film (Kodak, Rochester, NY).
Preparation of DD gel

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>6.84 g</td>
</tr>
<tr>
<td>Bis-acrylamide</td>
<td>0.34 g</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>TBE 10X</td>
<td>12 ml</td>
</tr>
<tr>
<td>Bidistilled water</td>
<td>120.8 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.025 ml</td>
</tr>
</tbody>
</table>

6.5 Analysis of differential bands

The differentially expressed bands were cut off from the gel, boiled in 100 µl water for 15 minutes and then reamplified by PCR. The PCR products were then cloned into pT-Adv vector (Clontech Laboratories, PaloAlto, CA) with the following ligation protocol:

<table>
<thead>
<tr>
<th></th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>3</td>
</tr>
<tr>
<td>10X Ligation buffer</td>
<td>1</td>
</tr>
<tr>
<td>pT-Adv (25 ng/µl)</td>
<td>2</td>
</tr>
<tr>
<td>Fresh PCR product (≈ 10 ng)</td>
<td>3</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10</td>
</tr>
</tbody>
</table>

The ligation products were incubated at 4°C overnight. The next day, the transformation was performed. 1-2 µl of each ligation product were mixed with *E.coli* competent cells,
incubated 30 minutes on ice, heated for 2 minutes in a 42°C water bath and placed again on ice for 4 minutes. Then, 350 µl of L-Broth (LB) medium (10 mg/ml NaCl, 10 mg/ml Tryptone Peptone, 5 mg/ml yeast extract) were added to each tube and incubated at 37°C for 1 hour. 50 µl of each transformation were plated on ampicillin (100 µg/ml) LB/X-Gal/IPTG plates with 30 µl X-Gal 50 mg/ml (Promega, Madison, WI) and 50 µl IPTG 1 M (Promega, Madison, WI). The plates were incubated for 18 hours at 37°C and shifted to 4°C for 3-4 hours for colour development. The presence of inserted fragments was verified by enzymatic digestion. The DD fragments were then isolated and used for Northern probe preparation, sequenced (University Core DNA & Protein Services, University of Calgary, Calgary, AB) and compared to Genbank Database.

7. Doubling time calculation

7.1 Doubling time

IEC-6 cells expressing different C/EBP isoforms were washed two times with PBS 1X and treated with 1 ml trypsin (Gibco-BRL, Burlington, ON). After 2 minutes at 37°C, 4 ml of DMEM containing 5% FBS were added. Then, using a hemacytometer, cells were counted and 10000 cells were plated in each well of a 24 well-plate. The next day, 100 or 200 µl trypsin were added to each well and the cells counted. The counting was repeated after 48 and 72 hours. The doubling time was calculated on a logarithmic graph. The experiment was done 4 times and standard deviation established.
7.2 Statistical analysis

Significance of the mean values was determined by Student's t-test. Statistic difference was fixed at 95% (p<0.05) limit.

8. DNA laddering

Different C/EBP expressing IEC-6 cells were plated in 100 mm dishes. At 90% confluence, the cells were washed two times with PBS 1X. Then, the cells were trypsinized and replated in non-adherent 100 mm bacterial dishes. After 24 hours, the cells were transferred in 15 ml tubes and centrifuged for 10 minutes at 2000 RPM at 4°C. The cells were then resuspended in 1 ml PBS 1X and centrifuged for 2 minutes at 2000 RPM at room temperature. Then, the cells were mixed with 600 μl cold apolysis buffer (10 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 0.5% Triton X-100) and vortexed for 30 seconds. After 30 minutes on ice, the tubes were centrifuged for 15 minutes at 4°C. The supernatant was transferred in another tube and three extractions with phenol-chloroform-isoamyl alcohol (25: 24: 1) were performed. Each extraction was followed by 30 seconds vortexing and 3 minutes centrifugation. Then, the upper phase was transferred in another tube and the DNA was precipitated overnight at room temperature with 1 ml 100% cold ethanol and 1.5 μl glycogen (20 μg/μl). The next day, after 15 minutes centrifugation at 4°C and 13000 RPM, the pellet was washed with 300 μl cold ethanol (70%). After another 4 minutes centrifugation at 4°C, the DNA containing pellet was resuspended in 20 μl TE/NaCl 50 mM (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 50 mM NaCl) and incubated overnight at room temperature. The next day, the DNA was treated with
RNAsé and incubated for 20 minutes at 37°C. The DNA laddering was verified on a 1.5% agarose gel with 1/10 volume of ethidium bromide and in TAE 1X buffer.
RESULTS

1. Effect of C/EBP isoform expression on the regulation of acute phase proteins and C/EBPs

1.1 Effect of C/EBP isoform expression on C/EBP endogenous protein levels

To determine the role of specific C/EBP isoforms in intestinal epithelial cells, we generated IEC-6 rat intestinal epithelial cell lines and populations stably expressing different C/EBP isoforms and dominant-negative C/EBPs, by retroviral infection and puromycin selection. The coding sequences for C/EBPa p42 and p30, C/EBPβ LAP and LIP and C/EBPδ, as well as the DNA-binding C/EBP dominant-negative 3hF and 4hF were cloned in the EcoRI site of the retroviral vector pBabepuro (Fig. 4). 10 clones were isolated for each infection. Nuclear extracts were prepared and analysed by electrophoretic mobility shift assays with a haptoA C/EBP DNA-binding site from the haptoglobin promoter (Pelletier et al., 1998; Yu et al., 1999). The results shown in Fig. 5A indicated an increase in DNA-binding activity with nuclear extracts obtained from C/EBPa p30, C/EBPβ LAP, C/EBPβ LIP and C/EBPδ expressing clones. Then, we wanted to determine the nature of the C/EBP isoforms in these complexes. To accomplish this, we performed supershift assays using specific antibodies against different C/EBP isoforms. As compared to empty vector nuclear extracts (puro1), C/EBPa, C/EBPβ and C/EBPδ isoforms were increased in C/EBPa p30, C/EBPβ LAP and LIP, and C/EBPδ nuclear extracts (Fig. 5B). In addition, we observed an increase in endogenous C/EBPβ isoforms in C/EBPa p30 and C/EBPδ nuclear extracts. Western blot analysis confirmed
this increase in both C/EBP LAP and LIP endogenous protein levels in C/EBPα p30 and C/EBPδ nuclear extracts (Fig. 6).

1.2 Effect of C/EBP isoform expression on the regulation of acute phase proteins and C/EBPs

We next assessed the effect of C/EBP isoforms on the expression of acute phase protein genes and C/EBPs, and verified the degree of responsiveness of these genes to known regulators, namely glucocorticoids (dexamethasone), cAMP (forskolin) and IL-1. Our results show the importance of C/EBP isoform expression in the regulation of haptoglobin. By Northern blot analysis, we observed that overexpression of C/EBPα p30, C/EBPβ LAP and C/EBPδ increased the basal haptoglobin mRNA levels (Fig. 7. control). Furthermore, the responsiveness of haptoglobin to dexamethasone, forskolin and IL-1 was increased in these C/EBP isoform overexpressing clones. Overexpression of the dominant-negative C/EBPβ LAP inhibited the induction of haptoglobin (Fig. 7). This dominant-negative C/EBPβ isoform dimerizes with other C/EBP isoforms and binds DNA, but lacks the transactivation domains (Descombes and Schibler. 1991).

We were also interested to verify the expression of another acute phase protein, the α-acid glycoprotein. We found that, in contrast to haptoglobin, the basal levels of α-acid glycoprotein were not increased by C/EBP isoform overexpression. In addition, the responsiveness of α-acid glycoprotein to dexamethasone was not affected in C/EBP isoform overexpressing clones, including the dominant-negative C/EBPβ LIP clones.
(Fig. 7). This suggests that, in contrast to other cell types, α-acid glycoprotein expression is not regulated by C/EBP isoforms in intestinal epithelial cells.

It has been previously suggested that C/EBPs were involved in their own regulation by an autoregulatory mechanism. This has been proposed for C/EBPα (Christy et al., 1991), C/EBPβ (Chang et al., 1995) and C/EBPδ (Yamada et al., 1998). We therefore verified their expression levels in the various clones. Our results show that overexpression of the various C/EBP isoforms or of the dominant-negative C/EBPβ LIP did not alter the mRNA levels of C/EBPβ and C/EBPδ in non-induced or in induced intestinal epithelial cells (Fig. 7).

To confirm the dominant effect of C/EBPβ LIP and the involvement of C/EBP isoforms in the regulation of haptoglobin expression, we established lEC-6 cell populations expressing the 3hF and 4hF dominant-negative constructs by retroviral infection. 3hF and 4hF are dominant-negative proteins that inhibit the DNA-binding activity and the function of C/EBP isoforms (Krylov et al., 1995). Overexpression of these dominant-negative proteins led to a decrease in C/EBP DNA-binding to the haptoA site in non-induced cells, as shown by electrophoretic mobility shift assays (Fig. 8A). This decrease was also observed following dexamethasone, IL-1 and forskolin treatment. By supershift assays, we demonstrated that the ability of the three C/EBP isoforms to bind to haptoA was reduced (Fig. 8B). While the dominant-negative constructs decreased the responsiveness of the haptoglobin gene to dexamethasone, IL-1 and forskolin, expression of C/EBPβ, C/EBPδ and α-acid glycoprotein was not altered, as shown by Northern blot
analysis (Fig. 9). This confirms that in intestinal epithelial cells, C/EBP isoforms are involved in the regulation of haptoglobin. In addition, induction of α-acid glycoprotein by dexamethasone is independent of C/EBP isoform expression in intestinal epithelial cells.

2. Effect of C/EBP isoform expression on cell growth and anoikis

2.1 Effect of C/EBP isoform expression on cell growth

We then analyzed the role of C/EBP on cell growth of intestinal epithelial cells. As in other cell types (Hendricks-Taylor and Darlington, 1995) the establishment of IEC-6 cell lines after two infections with a C/EBPα p42 expressing vector arrested cell growth. We first verified the doubling times of various C/EBP isoform expressing clones. As shown in Table 2, the doubling times of C/EBPα p30, C/EBPβ LIP and C/EBPδ clones were not significantly altered, as compared to empty vector infected clones. In contrast, the doubling time of C/EBPβ LAP clones was increased.

Next, we assessed the expression of various regulators of cell growth after serum induction. The results obtained by Western blot analysis show that pRb hyperphosphorylation and inactivation was delayed in C/EBPβ overexpressing clones, as compared to control cells (Fig. 10A), while the p42/p44 MAP kinases were normally induced in response to serum (Fig. 10B). C/EBPβ LAP overexpression also led to decreases in the expression of cyclin D isoforms and cyclin E, and to increases of the cyclin-dependent kinase inhibitor p27Kip1 expression levels (Fig. 11).
2.2 Effect of C/EBP isoform expression on anoikis

We further verified the role of the C/EBPβ LAP isoform on apoptosis of intestinal epithelial cells. Intestinal epithelial cells expressing different C/EBP isoforms were maintained in suspension for 24 hours and the integrity of nuclear DNA was determined. Most apoptotic cells contain degraded chromosomal DNA caused by endonuclease digestion (Caron-Leslie et al., 1991; Barry and Eastman, 1993; Peitsch et al., 1993). This can be visualised by gel electrophoresis as a DNA ladder. As shown in Fig. 12, cells expressing C/EBPβ LAP, and to a smaller extent C/EBPα p30, presented a reduced DNA fragmentation pattern in comparison with cells overexpressing other C/EBP isoforms. This suggests that C/EBPβ LAP overexpression may protect intestinal epithelial cells against anoikis.

2.3 Identification of differentially expressed genes

Another goal of my studies was to identify differentially expressed genes by comparing C/EBPβ LIP expressing cells and C/EBPβ LAP expressing cells. C/EBPβ LIP is the truncated form of C/EBPβ, which lacks the transactivation domains and functions as a dominant negative. By differential display method and using different sets of primers, we identified a set of differentially expressed genes. These genes were isolated and used for Northern analysis. Representative results are shown in Fig. 13. As compared to the C/EBPβ LIP clone (1), the C/EBPβ LAP clone (2) presented some differentially expressed genes (Fig. 13B, C, D, and E as compared to 13A). Then, these differentially expressed genes were sequenced; a series of similarities were found with genes from the
GenBank Database (Table 3). The protein matches for the sequences of the genes we found are indicated in Table 3 (Altschul et al., 1997).
The coding regions of C/EBPα p42 and p30, C/EBPβ LAP, C/EBPβ LIP and C/EBPδ were cloned in the EcoRI site of the retroviral vector pBabe puro. The DNA-binding C/EBP dominant-negative 3hF and 4hF sequences were PCR-amplified and subcloned in the same retroviral vector.
Figure 5. Composition of C/EBP DNA-binding complexes in IEC-6 cell lines overexpressing C/EBP isoforms.

(A) Nuclear extracts from representative empty vector (puro), C/EBPα p30, C/EBPβ LAP, C/EBPβ LIP and C/EBPδ selected clones were prepared and mixed with the haptoA C/EBP DNA-binding site labelled probe. DNA-protein complexes were separated from the free probe on a native polyacrylamide gel for electrophoretic mobility shift assays. (B) Supershift analysis. Nuclear extracts were incubated with the haptoA DNA-binding labeled probe without (-) or with antibodies against C/EBPα, C/EBPβ and C/EBPδ to determine the composition of the C/EBP complex. Incubation with the HNF1 antibody was used as a specificity control.
Figure 6. C/EBPβ endogenous protein levels are increased in C/EBPα p30 and C/EBPδ overexpressing cell clones.

40 µg of nuclear extracts isolated from representative empty vector (puro 1), C/EBPα p30, C/EBPβ LAP, C/EBPβ LIP and C/EBPδ selected clones were separated by SDS-PAGE and transferred to PVDF membranes for Western blot analysis for C/EBPα, C/EBPβ and C/EBPδ expression. The position of the different forms of C/EBPα (p42 and p30) and C/EBPβ (LAP and LIP) is indicated.
Figure 7. Pattern of expression of haptoglobin, α-acid glycoprotein and C/EBP isoforms in C/EBP overexpressing clones.

Total RNAs were isolated from empty vector (puro 1), C/EBPα p30, C/EBPβ LAP, C/EBPβ LIP and C/EBPδ selected clones treated for 24 hours without (control) or with 10^{-6} M dexamethasone, 1 µM forskolin or 10 ng/ml human recombinant IL-1. Equal amounts of RNA (40 µg) were electrophoresed and analysed sequentially by Northern blot with 32P-labeled haptoglobin, C/EBPβ, C/EBPδ, α-acid glycoprotein (AGP) and α-tubulin probes.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>puro1</td>
<td>p3012</td>
</tr>
<tr>
<td>Hapto</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/EBPβ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/EBPδ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Forskolin</th>
<th>IL-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>puro1</td>
<td>p3012</td>
</tr>
<tr>
<td>Hapto</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/EBPβ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/EBPδ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 8. Composition of C/EBP DNA-binding complexes in IEC-6 cell lines overexpressing dominant-negative proteins.

(A) Nuclear extracts were prepared from empty vector (puro 1), 3hF and 4hF dominant-negative protein selected cell lines treated without (1) or with 10⁻⁶M dexamethasone (2), 1 µM forskolin (3) or 10 ng/ml human recombinant IL-1 (4) for 24 hours, and mixed with the haptoA C/EBP DNA-binding site labelled probe. DNA-protein complexes were separated from the free probe on a native polyacrylamide gel for electrophoretic mobility shift assays. (B) Supershift analysis. Nuclear extracts from untreated empty vector (puro 1), 3hF and 4hF dominant-negative protein selected cell lines were incubated with the haptoA DNA-binding site labelled probe without (-) or with antibodies against C/EBPα, C/EBPβ and C/EBPδ to determine the composition of the C/EBP complex.
Figure 9. Pattern of expression of haptoglobin, α-acid glycoprotein and C/EBP isoforms in dominant-negative overexpressing clones.

Total RNAs were isolated from empty vector (puro), 3hF and 4hF dominant-negative protein selected cell populations treated for 24 hours with 10^{-6}M dexamethasone, 1 μM forskolin or 10 ng/ml human recombinant IL-1. Equal amounts of RNA (40 μg) were electrophoresed and analysed sequentially by Northern blot with ^{32}P-labeled haptoglobin, C/EBPβ, C/EBPδ, α-acid glycoprotein (AGP) and α-tubulin probes.
<table>
<thead>
<tr>
<th></th>
<th>Dexamethasone</th>
<th></th>
<th>IL-1</th>
<th></th>
<th>Forskolin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>puro</td>
<td>3hF</td>
<td>4hF</td>
<td>puro</td>
<td>3hF</td>
</tr>
<tr>
<td>Hapto</td>
<td><img src="Hapto" alt="Image" /></td>
<td><img src="Hapto" alt="Image" /></td>
<td><img src="Hapto" alt="Image" /></td>
<td><img src="Hapto" alt="Image" /></td>
<td><img src="Hapto" alt="Image" /></td>
</tr>
<tr>
<td>C/EPBβ</td>
<td><img src="C/EPB%CE%B2" alt="Image" /></td>
<td><img src="C/EPB%CE%B2" alt="Image" /></td>
<td><img src="C/EPB%CE%B2" alt="Image" /></td>
<td><img src="C/EPB%CE%B2" alt="Image" /></td>
<td><img src="C/EPB%CE%B2" alt="Image" /></td>
</tr>
<tr>
<td>C/EBPδ</td>
<td><img src="C/EBP%CE%B4" alt="Image" /></td>
<td><img src="C/EBP%CE%B4" alt="Image" /></td>
<td><img src="C/EBP%CE%B4" alt="Image" /></td>
<td><img src="C/EBP%CE%B4" alt="Image" /></td>
<td><img src="C/EBP%CE%B4" alt="Image" /></td>
</tr>
<tr>
<td>α-tubulin</td>
<td><img src="%CE%B1-tubulin" alt="Image" /></td>
<td><img src="%CE%B1-tubulin" alt="Image" /></td>
<td><img src="%CE%B1-tubulin" alt="Image" /></td>
<td><img src="%CE%B1-tubulin" alt="Image" /></td>
<td><img src="%CE%B1-tubulin" alt="Image" /></td>
</tr>
<tr>
<td>AGP</td>
<td><img src="AGP" alt="Image" /></td>
<td><img src="AGP" alt="Image" /></td>
<td><img src="AGP" alt="Image" /></td>
<td><img src="AGP" alt="Image" /></td>
<td><img src="AGP" alt="Image" /></td>
</tr>
</tbody>
</table>
Table 2. Growth characteristics of IEC-6 cells overexpressing C/EBP isoforms.

The experiment was done 4 times and standard deviation established. Statistic difference was fixed at 95% (p<0.05) limit.
<table>
<thead>
<tr>
<th>Cells</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>puro1</td>
<td>26.4±0</td>
</tr>
<tr>
<td>puro2</td>
<td>25.2±1</td>
</tr>
<tr>
<td>p30 11</td>
<td>28.5±1</td>
</tr>
<tr>
<td>p30 12</td>
<td>34.8±2</td>
</tr>
<tr>
<td>LIP1</td>
<td>23.7±1</td>
</tr>
<tr>
<td>LIP7</td>
<td>26.4±1</td>
</tr>
<tr>
<td>δ1</td>
<td>25.5±1</td>
</tr>
<tr>
<td>δ5</td>
<td>25.5±3</td>
</tr>
<tr>
<td>LAP1</td>
<td>52.2±7</td>
</tr>
<tr>
<td>LAP3</td>
<td>41.1±7</td>
</tr>
<tr>
<td>LAP4</td>
<td>43.2±7</td>
</tr>
<tr>
<td>LAP7</td>
<td>41.1±2</td>
</tr>
</tbody>
</table>
Figure 10. Time course of pRb phosphorylation and p42/p44 MAP kinase activation in control and C/EBPβ LAP overexpressing clones.

Control (non-infected IEC-6 population, empty vector infected cell clone puro1) and C/EBPβ LAP overexpressing clone 1 (LAP1) were serum-starved for 48 hours and stimulated with 5% FBS for 10 minutes. 2, 16 and 24 hours. 30 µg of whole cell protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis of pRb phosphorylation with antibodies recognizing the non- and the phosphorylated form of pRb (A) and p42/p44 MAP kinases phosphorylation, with antibodies against phosphorylated or total forms (B).
A-

<table>
<thead>
<tr>
<th></th>
<th>puro 1</th>
<th>IEC</th>
<th>LAP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>16h</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>24h</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B-

<table>
<thead>
<tr>
<th>FBS (min)</th>
<th>puro 1</th>
<th>LAP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
</tr>
<tr>
<td>10</td>
<td><img src="image12.png" alt="Image" /></td>
<td><img src="image13.png" alt="Image" /></td>
</tr>
<tr>
<td>120</td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
</tbody>
</table>

- pRb-P
- pRb
- p44-P
- p42-P
- p44
- p42
Figure 11. Time course of cyclin D1/2, cyclin E and p27Kip1 expression in control and C/EBPβ LAP overexpressing clones.

Control (empty vector infected cell clone puro1) and C/EBPβ LAP overexpressing clone 1 (LAP1) were serum-starved for 48 hours and stimulated with 5 % FBS for 2, 4, 8, 16 and 24 hours. 30 μg of whole cell protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis of cyclin D1/2, cyclin E and p27Kip1.
Figure 12. DNA laddering of different C/EBP isoforms expressing clones.

Cells expressing different C/EBP isoforms were plated on non-adherent bacterial dishes and maintained for 24 hours in suspension. The DNA was then isolated and separated on a 1.4% acrylamide gel.
Total RNAs were isolated from C/EBPβ LIP (1) and LAP (2) clones. Equal amounts of RNA (30 µg) were electrophoresed and analysed sequentially by Northern blot with α-[\(^{32}\)P]dCTP labelled probes obtained from differentially expressed genes by the differential display method. The arrows show the differentially expressed genes (B, C, D, E), as compared with non-differentially expressed genes (A).
Table 3. Gene similarities when compared to genes from the Genbank Database

DI-differentially expressed genes; NDI-nondifferentially expressed genes
<table>
<thead>
<tr>
<th></th>
<th>DI</th>
<th>NDI</th>
<th>Rat</th>
<th>Mouse</th>
<th>Identifiers</th>
<th>E value</th>
<th>Protein similarity</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A6-1</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td>AC024809</td>
<td>0.005</td>
<td>Ribonucleotide reductase, NRDI protein</td>
<td>3e-05</td>
</tr>
<tr>
<td>2A6-2</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td>dbEST Id: 7526499</td>
<td>0.079</td>
<td>Ribonucleotide reductase, NRDI protein</td>
<td>1e-05</td>
</tr>
<tr>
<td>1A1</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>dbEST Id: 7080905</td>
<td>e-108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A1-1</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>dbEST Id: 7080905</td>
<td>e-115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2G4-1</td>
<td>x</td>
<td></td>
<td>x</td>
<td>x</td>
<td>dbEST Id: 2316891</td>
<td>e-92</td>
<td>Voltage-gated potassium channel protein KQT-like 2</td>
<td>0.56</td>
</tr>
<tr>
<td>2G5-1</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td>dbEST Id: 4789420</td>
<td>0.54</td>
<td>Probable aromatic acid Decarboxylase</td>
<td>1.7</td>
</tr>
<tr>
<td>2G5-2</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td>dbEST Id: 5784256</td>
<td>1.8</td>
<td>No significant similarity found</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

1. C/EBP isoforms are involved in the regulation of the acute phase protein gene haptoglobin

The results of my work show that C/EBP isoforms are involved in the regulation of the acute phase protein gene haptoglobin in intestinal epithelial cells. Overexpression of C/EBPα p30, C/EBPβ LAP and C/EBPδ independently leads to increased basal levels of haptoglobin. Furthermore, the level of haptoglobin mRNA induction in response to glucocorticoids, forskolin and IL-1 is increased when C/EBP isoforms are expressed. This confirms previous results obtained in our laboratory showing that the C/EBPβ and C/EBPδ isoforms are the most potent activators of haptoglobin expression (Pelletier et al., 1998; Yu et al., 1999). In addition, these results show that C/EBPα also plays an important role in the regulation of haptoglobin in intestinal epithelial cells. Burgess-Beusse and Darlington (1998), by studying the neonatal acute phase response to inflammation in C/EBPα knock-out mice, demonstrated that C/EBPα expression was required for haptoglobin expression in liver as opposed to lung. In our case, it is surprising that C/EBPα p30 is so effective. The two C/EBPα isoforms, p42 and p30, arise through the use of different translational initiation sites (Lin et al., 1993; Ossipow et al., 1993). The N-terminal domain of C/EBPα contains three separate transactivation domains. In contrast, C/EBPα p30 lacks two transactivation domains and presents a decreased transactivation potential as compared to the full-length C/EBPα p42 protein. It is not known whether the capacity of C/EBPα p30 to activate haptoglobin expression
depends on its ability to increase the endogenous levels of C/EBPβ in IEC-6 cells when overexpressed.

Overexpression of the dominant-negative transactivation repressor C/EBPβ LIP leads to a decrease of basal haptoglobin mRNA levels. Furthermore, the responsiveness of haptoglobin to dexamethasone, IL-1 and forskolin was decreased in these clones. The expression of the dominant-negative DNA-binding inhibitors, 3hF and 4hF, negatively influence the induction of haptoglobin by dexamethasone, IL-1 and forskolin. By binding to C/EBP isoforms, 3hF and 4hF prevent the binding of these transcription factors to their DNA-binding sites. C/EBP isoforms are induced during inflammation by inflammatory stimuli such as bacterial lipopolysaccharide and by recombinant cytokines such as IL-6, IL-1 and TNF-α (Akira and Kishimoto, 1997). The acute phase proteins are also induced during inflammation. Here we show that C/EBP isoforms are involved in the regulation of acute phase protein gene haptoglobin and confirm the importance of these transcription factors in the control of haptoglobin in intestinal epithelial cells.

2. C/EBP isoforms are not involved in the regulation of α-acid glycoprotein in intestinal epithelial cells

In contrast, C/EBP isoforms are not involved in the regulation of α-acid glycoprotein by dexamethasone in intestinal epithelial cells. Overexpression of dominant-negative C/EBPs, affecting either the transactivation or the DNA-binding potential, does not inhibit α-acid glycoprotein induction by dexamethasone. In addition, overexpression of
specific C/EBP isoforms does not increase basal α-acid glycoprotein mRNA levels. This indicates that the main regulator of α-acid glycoprotein expression is the glucocorticoid receptor, in contrast to α-acid glycoprotein regulation in rat liver or in other species. Indeed, glucocorticoids act through a minimal steroid responsive unit composed of a glucocorticoid receptor DNA-binding site overlapping C/EBP DNA-binding sites in the proximal α-acid glycoprotein promoter (Fournier et al., 2000). In rat liver and in other species, a strong cooperation between the glucocorticoid receptor and C/EBPs is necessary for hormone induction. Our results suggest that this cooperation is not required for α-acid glycoprotein regulation by glucocorticoids in intestinal epithelial cells.

3. C/EBP isoforms are not subject to autoregulation in IEC-6 cells

It has been shown that C/EBP isoforms are subject to autoregulatory mechanisms in other cell types, as assessed by transient transfection studies. For example, the mouse C/EBPα promoter is autoregulated by C/EBPα and/or related factor(s) through binding to promoter elements in adipocytes (Christy et al., 1991) and liver cells (Legraverend et al., 1993). The human C/EBPα promoter in hepatoma cells is indirectly autoregulated via stimulation by C/EBPα of binding of a ubiquitous factor named upstream stimulatory factor (USF) to the promoter element (Timchenko et al., 1995). Autoregulatory mechanisms have also been described for C/EBPβ (Chang et al., 1995) and C/EBPδ (Yamada et al., 1998). In contrast to other cell types, overexpression of specific C/EBP isoforms or dominant-negative forms of C/EBP did not affect C/EBP isoform mRNA levels. Furthermore, C/EBPα protein levels were not affected in C/EBP isoform
overexpressing cell lines. These results suggest that C/EBP isoforms are not subject to autoregulation in IEC-6 intestinal epithelial cells.

The intriguing increase in C/EBP DNA-binding activity and in C/EBPβ LAP and C/EBPβ LIP endogenous protein levels following C/EBPα p30 or C/EBPδ overexpression does not appear to involve transcriptional autoregulatory mechanisms. Though little is known of the mechanisms involved in dimerization of C/EBP isoforms in the cell. It is possible that dimers may be more stable than monomers. In this case, increases in one isoform may alter the steady-state levels of dimers by increasing the probability of forming dimers, thereby increasing the stability of the C/EBP isoform pool. Alternatively, C/EBP isoforms may compete with other proteins known to interact with C/EBPs with less affinity. For example, C/EBPα interacts with the transcription factor NF-κB (Yamada et al., 1998). Increases in C/EBPα protein levels may favour the formation of C/EBP dimers as opposed to C/EBPα-NF-κB dimers, thus increasing overall C/EBP DNA-binding activity. This hypothesis could be verified by immunoprecipitation-electrophoretic mobility shift assay (IP-EMSA). For example, using a specific antibody against NF-κB we could observe a decrease in NF-κB binding activity in intestinal epithelial cells overexpressing C/EBPα. Hence, this decrease in NF-κB binding may lead to the formation of C/EBP dimers and to a increase in C/EBP binding activity.
4. C/EBPβ LAP overexpression leads to a G1/S delay of intestinal epithelial cells

It has been shown that C/EBPα p42 overexpression leads to growth arrest in other cell types (Hendricks-Taylor and Darlington, 1995). C/EBPα-dependent cell growth arrest depends on the transcriptional regulation of the cyclin-dependent kinase inhibitor p21 (Timchenko et al., 1996) or its stabilization at the protein level (Timchenko et al., 1997) in different cell lines. More recently, C/EBPα has been shown to inhibit cell growth by direct repression of E2F-mediated transcription (Sanderson and Walker, 2000) and may disrupt E2F-p107 complexes (Timchenko et al., 1999). According to our data, we found that establishment of IEC-6 cell lines after infection with a C/EBPα p42 expressing vector arrested cell growth. The mechanisms involved in C/EBPα-dependent growth arrest in IEC-6 cells remain to be discovered.

We have shown that C/EBPβ LAP overexpressing clones presented an increased doubling time, as compared to C/EBPα p30, C/EBPβ LIP or C/EBPδ expressing clones. Furthermore, C/EBPβ LAP expressing cells presented a delayed hyperphosphorylation of pRb in response to serum and decreased levels of cyclin D1/2 and cyclin E. The levels of the cyclin-dependent kinase inhibitor p27Kipl were increased, while the p42/p44 MAP kinases were normally induced in response to serum. It has previously been shown that C/EBPβ inhibits hepatoma cell proliferation (Buck et al., 1994). In contrast, in primary hepatocytes, C/EBPβ is required for hepatocyte proliferation induced by TGFα (Buck et al., 1999). Our results suggest that C/EBPβ LAP overexpression leads to a G1/S delay of
intestinal epithelial cells. The exact mechanisms involved in this growth arrest remain to be determined.

5. C/EBPβ LAP overexpression seems to protect IEC-6 cells from apoptosis

Cell number homeostasis reflects a balance between cell proliferation and apoptosis. Apoptosis that occurs in cells detached from the matrix represents anoikis. Anoikis in vivo prevents detached cells from reattaching to new matrices and growing dysplastically (Frisch and Ruoslahti, 1997). Anoikis occurs in normal skin (Polakowska et al., 1994), in colonic epithelial tissues (Hall et al., 1994) and in the involuting mammary gland (Boudreau et al., 1995). The loss of integrin-mediated cell-matrix contact induces anoikis in certain cell types (Meredith et al., 1993). Cell-cell interactions also play important roles in protecting the cells against anoikis (Frisch and Ruoslahti, 1997). The expression levels of antiapoptotic proteins, like Bcl-2, Bcl-xL or proapoptotic factors (Bax, Bak, Bid, etc.) determine the sensitivity of cells to apoptosis. Besides, a series of protein kinases (FAK, AKT, PI3K) are involved in the signalling pathways responsible for the living status of the cells (Frisch and Ruoslahti, 1997).

It has been shown that C/EBPβ and C/EBPδ play important roles in the regulation of mammary epithelial cell apoptosis (Gigliotti and DeWill, 1998; O'Rourke et al., 1999). Furthermore, NF-M (chicken C/EBPβ) induces apoptosis in hematopoietic progenitor cell lines (Müller et al., 1995). In contrast, we have found that intestinal epithelial cells overexpressing C/EBPβ LAP present a reduced DNA fragmentation as compared to cells
overexpressing other C/EBP isoforms. This suggests that overexpression of C/EBPβ LAP may protect the cells against anoikis. The mechanisms involved in this protection in not known and remains to be determined.

6. Identification of differentially expressed genes

For the identification of DD genes, two C/EBP clones were used: one overexpressing C/EBPβ LIP, the other overexpressing C/EBPβ LAP. C/EBPβ LIP is the truncated form of C/EBPβ. It lacks the transactivation domains and functions as a dominant-negative. C/EBPβ LIP forms dimers with other C/EBP isoforms, binds DNA, but cannot activate gene transcription (Descombes and Schibler, 1991). On the other hand, C/EBPβ LAP is a potent transactivator. It is involved in the regulation and differentiation of adipocytes and hepatocytes (Cao et al., 1991; Williams et al., 1991), in the mediation of the acute phase response in hepatocytes (Poli, 1998) and intestinal epithelial cells (Boudreau et al., 1997; Yu et al., 1999; Désilets et al., 2000). It is also involved in the G1/S delay of intestinal epithelial cells. By differential display method, we isolated a series of genes expressed in C/EBPβ LAP, as opposed to C/EBPβ LIP. These genes were isolated and sequenced. The comparison of their nucleotide sequence to GenBank Database (nucleotide query-protein db) led us to the identification of a series of proteins. The genes that encode these proteins may represent putative targets of C/EBP family of transcription factors and may help us to understand their roles in intestinal epithelial cells.
One of the major criticism of the DD technique has been the high number of false positives obtained. To avoid these, attention in several areas is important, including use of high-quality RNA, reduction and control of intersample variability in the DD-PCR reaction or use of a reliable assay to confirm differential expression. Intersample variability could be minimized using cell populations overexpressing C/EBP isoforms, instead of clones.
CONCLUSIONS AND PERSPECTIVES

Until now, it has been shown that C/EBP isoforms are involved in the regulation of many cellular processes in different tissues. However, their exact roles in intestinal epithelial cells is still poorly understood. The principal aim of this research was therefore the identification of C/EBP functions in these cells.

In order to understand the role of C/EBP isoforms in the expression of some target genes, we generated rat intestinal epithelial cell lines (IEC-6) overexpressing different C/EBP isoforms or dominant-negative C/EBPs. First, we found that C/EBP isoforms are involved in the regulation of the acute phase response gene haptoglobin in intestinal epithelial cells. Overexpression of C/EBPα p30, C/EBPβ LAP and C/EBPδ led to increases in basal levels of haptoglobin and its responsiveness to dexamethasone, IL-1 and forskolin. On the other hand, overexpression of the dominant-negative transactivation repressor C/EBPβ LIP or dominant-negative DNA-binding inhibitors 3hF and 4hF, led to a decrease in haptoglobin expression in response to glucocorticoids, IL-1 and forskolin. However, other acute phase proteins are not under the control of C/EBP isoforms. We have shown that C/EBP isoforms are not involved in the regulation of the acute phase protein gene α-acid glycoprotein in intestinal epithelial cells. When we overexpressed specific C/EBP isoforms, no increase was observed in basal levels of α-acid glycoprotein. In addition, the responsiveness of α-acid glycoprotein to glucocorticoids was not affected when dominant-negative C/EBPs or C/EBP isoforms were overexpressed. This indicates that the major regulator of α-acid glycoprotein expression
in intestinal epithelial cells is the glucocorticoid receptor, as opposed to C/EBP proteins. Finally, overexpression of C/EBP isoforms or dominant-negative C/EBPs did not alter the mRNA levels of C/EBPβ and C/EBPδ in non-induced or in induced intestinal epithelial cells. This demonstrates that C/EBP isoforms are not subject to autoregulatory mechanisms in intestinal epithelial cells.

Further, we focused on the roles of C/EBP isoforms in intestinal cell processes. In this regard, we have shown that C/EBP expression affects cell proliferation of intestinal epithelial cells, as the doubling time of C/EBPβ LAP clones was increased. In this regard, we found that pRb inactivation in response to serum was delayed in C/EBPβ LAP clones. cyclin D1/2 and cyclin E expression was decreased, while p27kip1 expression was increased. By contrast, the MAP kinase p42/p44 was normally induced in response to serum. Lately we have found that C/EBP isoforms α and β interact with the E2F4 transcription factor in intestinal epithelial cells (Gheorghiu et al., 2001), process which affects the proliferation of these cells. The mechanisms by which these two C/EBP isoforms negatively alters the transactivation potential of E2F complexes remain to be determined. Then, we verified the influence of C/EBPβ LAP overexpression on intestinal epithelial cell death. We observed that, after maintaining the cells for 24 hours in suspension, those that overexpress C/EBPβ LAP have a reduced DNA fragmentation. This suggests that C/EBPβ LAP overexpression may protect the intestinal epithelial cells against anoikis. We do not know by which mechanism C/EBPβ LAP acts. It would be interesting to analyse (by Western blot) the pattern of anti-apoptotic (Bcl-2, Bcl-xL) and
pro-apoptotic (Bax, Bad, Bid) factors, as well as other factors that may intervene in this protection (e.g. specific protein kinases).

In order to discover other functions of C/EBP isoforms in intestinal epithelial cells, we also need to identify new targets of these transcription factors. In this direction, we performed differential display assays, a method which allows us to isolate differentially expressed genes. Thus, comparing the cells overexpressing C/EBPβ LIP with those overexpressing C/EBPβ LAP, we were able to isolate a series of new C/EBP target genes. However, further analyses should be performed to identify them and to understand their roles. Also, it would be interesting to find out if the expression of these genes is ubiquitous or tissue-specific. In addition, using the same method and another set of primers, novel targets of C/EBP family of transcription factors in intestinal epithelial cells could be identified and analyzed. Finding new targets would help us to better understand the roles of C/EBP isoforms in intestinal epithelial cells.

Finally, another interesting approach would be to obtain knockout mouse models for the three C/EBP isoforms I have studied. Knock-out mice for different C/EBP isoforms have already been produced, as Table 1 indicates. However, the effect of lacking these isoforms at the intestine level has not yet been studied. Thus, these mouse models would allow us to understand how the absence of C/EBPα, β or δ isoforms affects the acute phase response in the intestine and the resolution of intestinal inflammation. Besides, it would help us to confirm our results in vivo.
ACKNOWLEDGEMENTS

First, I would like to thank my research director, Dr. Claude Asselin, for giving me the chance to continue with my graduate studies and for his continuous encouragement and support.

I would also like to thank Dr. Nathalie Rivard and Dr. Pierre Vachon for their collaboration and valuable discussions during my studies.

I thank my laboratory colleagues, Antoine Désilets from whom I had learned many laboratory techniques, Mylène Blais for her help, especially in the last part of my studies, Amy Svotelis, Gabriela Ghinet, Sébastien Mongrain, Michele Sullivan, Jérome Barbier and Noémie Juaire. A special thank I would like to address to Claude Deschênes for helping me with the experimental work concerning the cell cycle regulators.

Finally, I thank all the professors, technicians, secretaries, and students of the Department for their help and friendliness.
REFERENCES


Hendricks-Taylor L. R. and Darlington G. J. (1995) Inhibition of cell proliferation by C/EBPα occurs in many cell types, does not require the presence of p53 or Rb, and is not affected by large-T antigen. Nucl. Acids Res. 23. 4726-4733.


involves autoregulation and several ubiquitous transcription factors. Nucleic Acids Res. 21, 1735-1742.


Roman C., Platero J. S., Shuman J., and Calame K. (1990) Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. Genes Dev. 4, 1404-1415.


Williams S. C., Baer M., Dillner A. J. and Johnson P. F. (1995) CRP2 (C/EBPβ) contains a bipartite regulatory domain that controls transcriptional activation. DNA binding and cell specificity. EMBO J. 14, 3170-3183.

