Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein

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This paper presents the results of experiments that determine the chromosomal location of the mouse gene encoding CCAAT/enhancer binding protein (C/EBP) and measure its expression as a function of tissue type and temporal period of development in mice and rats. Three alleles of the C/EBP gene were identified according to restriction fragment length polymorphisms. The strain distribution pattern of the three alleles was determined in recombinant inbred mouse strains and compared to that of other mouse genes. These results mapped the gene to a position within 2.5 centimorgans (cM) of the structural gene encoding glucose phosphate isomerase on chromosome 7 of the mouse. The expression pattern of the C/EBP gene was studied by a combination of nucleic acid hybridization and antibody staining assays. High levels of C/EBP mRNA were observed in tissues known to metabolize lipid and cholesterol-related compounds at uncommonly high rates. These included liver, fat, intestine, lung, adrenal gland, and placenta. More detailed analysis of two of these tissues, liver and fat, showed that C/EBP expression was limited to fully differentiated cells. Moreover, analysis of the temporal pattern of expression of C/EBP mRNA in two tissues, liver and intestine, revealed a coordinated induction just prior to birth. These observations raise the possibility that the synthesis of C/EBP may be responsive to humoral factors and that modulation in C/EBP expression might mediate coordinated changes in gene expression that facilitate adaptive challenges met during development or during the fluctuating physiological states of adult life.

CCAAT/enhancer binding protein (C/EBP) is a heat-stable, sequence-specific DNA-binding protein first purified from rat liver nuclei (Graves et al. 1986; Johnson et al. 1987). This protein binds avidly to several different *cis*-regulatory DNA sequences commonly associated with viral genes transcribed by RNA polymerase II. Recent evidence has indicated that C/EBP also binds to regulatory DNA sequences associated with cellular genes that encode mRNA (Lichtsteiner et al. 1987; Costa et al. 1988). Because C/EBP binds to DNA sequences that tend to play a positive role in gene expression, we anticipated that it would constitute a transcriptional regulatory protein. Evidence favoring this interpretation has emerged recently from transfection studies using a recombinant DNA copy of the C/EBP gene (A. Friedman, E. Landschulz, and S. McKnight, in press).

A long-standing goal of studies on cellular factors that bind to viral genes has been to understand the regulatory roles played by these proteins in the context of the host cell or organism. How general is the action of a protein such as C/EBP? Does it bind to many or all genes that encode mRNA? Is it ubiquitous in its cell type distribution? Once the answers to these questions are known, it may be possible to find physiologic relationships between certain genes or cell types that use this protein. Having developed molecular reagents specific to C/EBP (antibodies and recombinant DNA copies of the gene and cDNA), we have now been able to study its tissue and cell type distribution as a function of development.

Results

Chromosomal location of the mouse gene encoding C/EBP

Southern blots containing mouse DNAs obtained from nine different inbred strains were probed with radioac-

tive C/EBP DNA. The probe DNA fragment corresponded to a 1.5-kb cDNA that covered slightly more than half of the 2.7-kb C/EBP mRNA (Landschulz et al. 1988b). The mouse genomic DNA samples were digested with the restriction endonucleases BamHI, PstI, KpnI, and EcoRV. As shown in Figure 1, each lane of the resulting autoradiogram exhibited only one hybridizing fragment. The intensity of the radioactive bands was consistent with the notion that the C/EBP gene is present as a single copy per haploid genome equivalent. Similar interpretations have been reported elsewhere from studies of the rat gene that encodes C/EBP (Landschulz et al. 1988b). The size of the radioactive band generated by either KpnI (20 kb) or EcoRV (12 kb) was the same in each of the nine inbred strain DNAs examined (data not shown). However, BamHI and PstI gave evidence of restriction fragment length polymorphisms (RFLPs) between the various inbred strains, allowing definition of three alleles of the gene encoding C/EBP (Fig. 1). We named the gene Cebp and designated its three alleles $Cebp^a$, $Cebp^b$, and $Cebp^c$ (Table 1).

We were able to determine the chromosomal location of Cebp by using the PstI RFLP to type recombinant inbred (RI) strains for the presence or absence of the $Cebp^b$ allele. Screening 26 B × D (C57BL/6J × DBA/2J) RI strains and 7 SW × L (SWR/J × C57L/J) RI strains showed no recombination (0 of 33) between the Cebp and glucose phosphate isomerase-1 (Gpi-1) loci. Thus, Cebp is located on chromosome 7 and, at the upper 95% confidence limit, within 2.5 centimorgans (cM) of Gpi-1. We also screened 18 AK × L (AKR/J × C57L/J) RI

strains and found no recombination with the DNA polymorphic fragment D7Rp2, which is located distal to Gpi-1 on mouse chromosome 7. Among the 26 B × D RI strains, recombination occurred between Cebp and D7Rp2 in two strains (14 and 32). Thus, in the 44 strains typed for D7Rp2, there were two recombination events, indicating that Cebp is located within 5 cM of D7Rp2 at the upper 95% confidence limit.

Tissue distribution of C/EBP mRNA in adult mice and rats

The tissue distribution of C/EBP mRNA was investigated by several methods of filter hybridization. Total cellular RNA was isolated from 10 different adult mouse tissues and 7 adult rat tissues. For each species, organs were harvested from age-matched males from an inbred strain and pooled prior to RNA extraction (see Materials and methods). Animals were sacrificed at comparable times of the day after being maintained on standard chow diets and 12-hr light cycling schedules. Figure 2 shows a Northern blot used to examine the concentration of C/EBP mRNA in eight mouse tissues. Radioactive bands corresponding to the 2.7-kb C/EBP mRNA (Landschulz et al. 1988b) were observed readily in two tissues (brown fat and liver). Significantly lower levels of C/EBP mRNA were observed in three additional tissues (small intestine, lung, and kidney), and little or no evidence of hybridizing mRNA was observed in brain, testes, and spleen.

The same eight RNA samples, along with two addi-
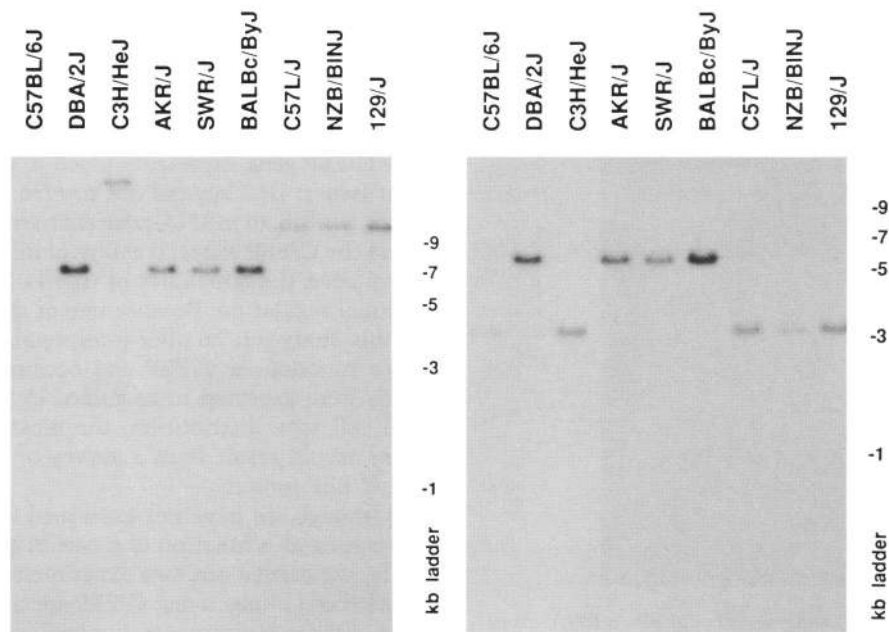
Figure 1. Southern blot analysis of *Cebp* alleles present in various inbred strains of mice. DNA samples from nine inbred strains were digested with either the restriction endonuclease *BamHI* (*left*) or *PstI* (*right*). Southern blots of these DNAs were hybridized to a ^{32}P-labeled C/EBP probe (Materials and methods). The autoradiograms reveal a single band present in each strain at the size indicated by the kilobase pair (kb) ladder. The kb ladder was generated using *HindIII*-digested λ DNA and *HaeIII*-digested πX174 DNA as markers.

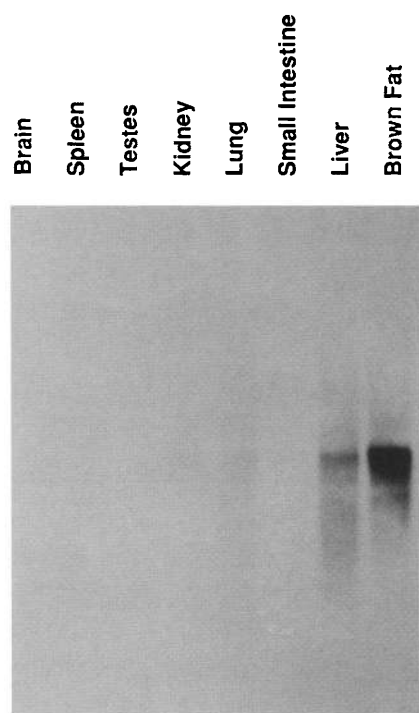**Table 1.** *Identification of three mouse C/EBP (Cebp) alleles*

| Group | Inbred strain | Restriction fragment size (kb) | | Allele |
		BamHI	PstI	
I	C57BL/6J			
	C3H/HeJ	15	3.3	Cebp^a
II	C57L/J			
	NZB/BINJ			
	129/J	9.4	3.3	Cebp^b
III	AKR/J			
	BALB/cByJ			
	DBA/2J			
	SWR/J	6.2	6.2	Cebp^c

DNA from nine inbred mouse strains was digested with either *BamHI* or *PstI*, Southern blotted, and hybridized to a rat C/EBP probe. The size of the single band found in each lane of the autoradiogram then was determined. Each of the three *Cebp* alleles was identified by the RFLPs shown.

tional samples (derived from white fat and skeletal muscle), were analyzed by dot-blot hybridization to facilitate a more quantitative comparison of mRNA abundance among the various tissues. Aliquots of 2 and 4 μg of each RNA were applied to a nylon filter (Materials and methods). C/EBP mRNA then was detected by hy-


Figure 2. Northern blot analysis of C/EBP mRNA from various tissues of adult male BALB/cByJ mice. Total cellular RNA was isolated from eight different tissues and 10 μg of each sample was electrophoresed on a denaturing agarose gel. A Northern blot of the gel was hybridized to a ³²P-labeled C/EBP probe (Materials and methods), generating the autoradiogram that is shown.

bridizing the filter to the same radioactively labeled C/EBP cDNA probe that was used in the Southern and Northern blot assays shown in Figures 1 and 2. After hybridization and washing, the nylon filter was exposed to X-ray film (Fig. 3A, right). The autoradiographic exposure was scanned by densitometry to measure differences in C/EBP mRNA concentrations (see Materials and methods). To ensure that consistent amounts of RNA remained on the filter after hybridization, the filter was examined by illumination with UV light (Fig. 3A, left). The results of these quantitations (Fig. 3B) provided results that were concordant with those generated by Northern blot analysis (Fig. 2). The C/EBP hybridization signal produced from liver RNA was arbitrarily set at 100%. Both brown and white fat RNA samples exhibited significantly higher levels of C/EBP mRNA than the liver standard, which was, in turn, slightly greater than lung and small intestine. According to these quantitations, kidney, skeletal muscle, brain, testes, and spleen appeared to contain relatively low amounts of C/EBP mRNA.

Similar experiments were carried out on RNA samples derived from seven adult rat tissues: five that had been examined in mice (liver, lung, small intestine, kidney, and brain) and two that had not (placenta and adrenal gland). Quantitations of dot-blot hybridization assays again showed C/EBP mRNA concentrations to be high in liver, low in brain, and intermediate in lung, small intestine, and kidney (Fig. 3B). RNA prepared from placentas harvested during the last day of gestation exhibited C/EBP mRNA levels 1.75-fold higher than that of adult rat liver, and adrenal gland RNA showed an intermediate level of the C/EBP transcript.

Immunological localization of C/EBP protein in adult liver tissue

The filter hybridization assays that were used to monitor C/EBP gene expression failed to address two important issues: (1) They did not resolve the identity of cell types, within an mRNA-positive tissue, that actively express the C/EBP gene; (2) assays of mRNA concentration leave open the possibility of translational or post-translational regulation. Because one of the major objectives of this study was to offer interpretations of the physiologic function of C/EBP and because such interpretations were expected to be guided by assays of its tissue and cell type distribution, the most valuable observations would result from a survey of the distribution of the C/EBP protein.

Although we have not examined C/EBP protein concentration as a function of tissue or cell type systematically, we carried out two experiments that begin to address these issues using C/EBP-specific antibodies. The first of these experiments involved in situ localization of C/EBP protein in squashed preparations of adult mouse liver. The second, which will be presented in a subsequent section, involved Western blot analysis of C/EBP concentration as a function of fat cell differentiation. As shown in Figure 4, antibodies specific to a 14-amino-acid
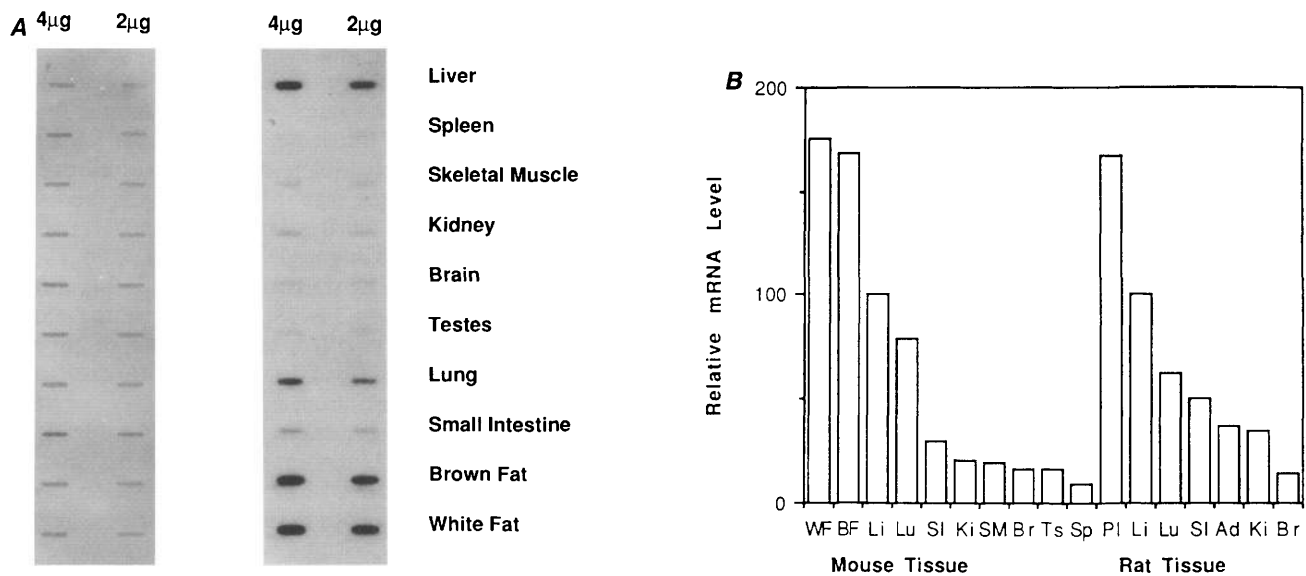
Figure 3. Distribution of C/EBP mRNA in adult mouse and rat tissues. Total cellular RNA was isolated from various tissues, and slot blots were prepared and hybridized to a ^{32}P-labeled C/EBP probe (Materials and methods). (A) A photograph of a slot blot using a UV transilluminator as an excitation light source is shown (left). The UV shadow observed in each slot results from the presence of either 2 or 4 μg of RNA. An autoradiogram of the same slot blot is shown after hybridization with a ^{32}P-labeled probe specific to the C/EBP mRNA (right). (B) The relative concentration of C/EBP mRNA in mouse tissues was determined by densitometric scanning of the autoradiogram shown in A (right). The C/EBP mRNA concentrations in liver was arbitrarily set at 100. The relative concentrations of C/EBP mRNA in rat tissues were determined using another slot blot (Materials and methods).

epitope within the C/EBP polypeptide (Landschulz et al. 1988b) were found to react with an antigen limited to the large, hepatocyte nuclei of adult liver. Aside from a standard series of control staining reactions (see Materials and methods), two additional observations favored the validity of this cytological assay. First, the reactivity specific to hepatocyte nuclei was blocked by a synthetic peptide corresponding to the epitope (data not shown).

Second, the same pattern of staining was observed using an antibody specific to a different epitope located within the carboxy-terminal 15 amino acids of C/EBP (data not shown).

Variable levels of C/EBP mRNA in adult mouse liver

Measurements of C/EBP mRNA concentration in various rat and mouse tissues indicated that the C/EBP
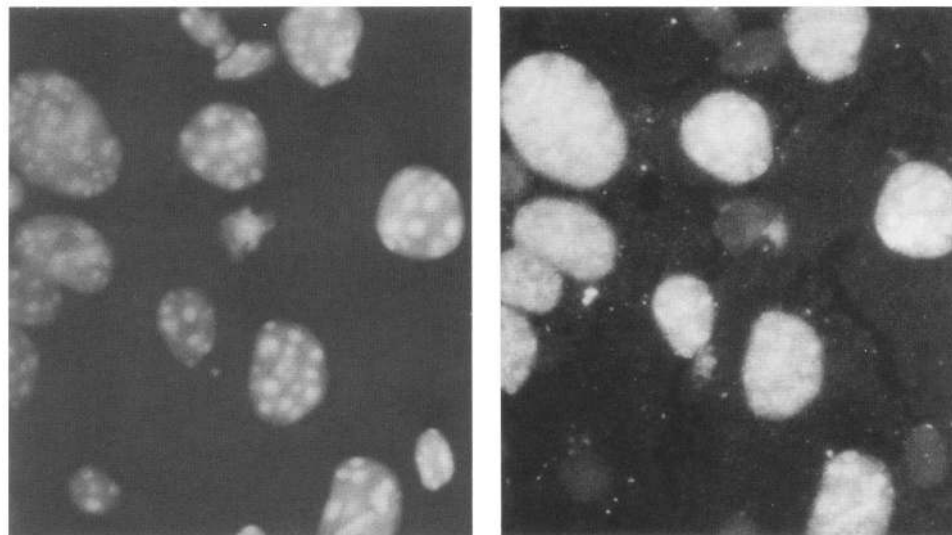
Figure 4. Immunofluorescent localization of C/EBP protein in adult liver tissue. Mouse liver tissue was prepared and stained for fluorescence microscopy, as described in Materials and methods. (Left) Nuclei localization by the DNA-specific fluorescent stain DAPI; (right) localization of C/EBP protein by immunofluorescent staining. Notice that C/EBP staining is restricted to the large parenchymal cell (hepatocyte) nuclei. Magnification, 340×.

transcript, although present at relatively high levels in mouse and rat liver, was also present in a variety of other tissues. However, because tissue was pooled from several animals prior to RNA extraction, it was not possible to determine whether there was animal-to-animal variation in C/EBP mRNA concentration. To begin to address this question, we measured C/EBP mRNA concentration in livers obtained from individual 8-week-old male BALB/cByJ mice. Prior to sacrifice, animals had been housed in separate cages within the same section of a mouse room for a 3-day period of adaptation with a standard chow diet. The only variable not standardized was the time of day the individual animals were killed. To *maximize* any contribution that time of day might have on C/EBP mRNA levels, the mice were sacrificed every 4 hr over a 3-day period. Much to our surprise, up to a fivefold variation in hepatic C/EBP mRNA concentration was observed between these genetically uniform animals. Interestingly, when the same RNA samples were probed with a rat serum albumin cDNA, comparable differences (up to threefold) were noted to correlate significantly ($p = 0.001$) with C/EBP mRNA concentration (Fig. 5). The significance and origins of fluctuation in C/EBP mRNA concentration are unclear. However, the observed correlation between C/EBP and albumin mRNA levels may be interpretable (see Discussion).

Developmental regulation of C/EBP mRNA in liver and intestine

The factors that modulate C/EBP expression in vivo are unknown. As an initial approach to study this problem,
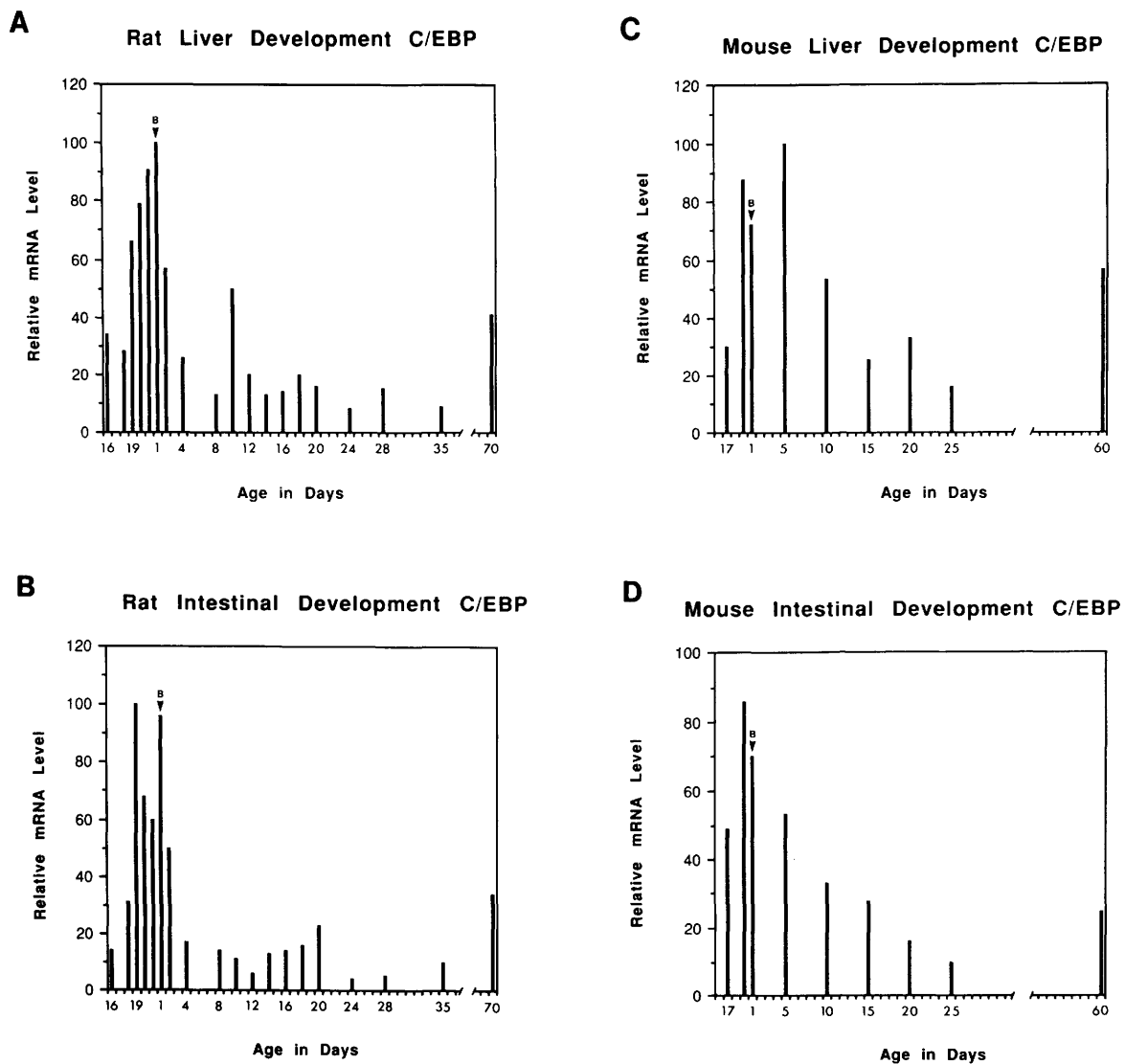
we monitored mRNA accumulation in two tissues as a function of developmental stage. Figure 6 summarizes the developmental changes in C/EBP mRNA accumulation observed within Sprague–Dawley rat and BALB/cByJ mouse liver and intestine. Total cellular RNA was isolated from the pooled livers or small intestines of animals at various ages (see Materials and methods). Samples were taken during late gestation, at various times during the suckling period (postnatal days 1–12), at the suckling/weaning transition (days 13–14), during the weaning phase (days 14–28), and from young adults. RNA was extracted, purified, and subjected to dot-blot hybridization, as described in Figure 3, to quantitate C/EBP mRNA concentration.

C/EBP mRNA was detectable in liver RNA during the sixteenth day of the 22-day rat gestation period and the seventeenth day of the 21-day mouse gestation period (see Fig. 6A and C). In both mammalian species, hepatic levels of C/EBP mRNA were observed to rise during the remainder of fetal life, reaching a peak during the early postnatal period. A subsequent fall was noted during the suckling period. Liver C/EBP mRNA levels varied less than twofold during the weaning phase, as animals change from the relatively high fat diet of mother's milk to the high carbohydrate diet of standard chow.

A similar pattern of C/EBP expression also was encountered in developing intestinal tissue of rats and mice (see Fig. 6B, D). In both species, C/EBP mRNA was detectable in fetal intestine, increased markedly in concentration during late gestation and the early suckling period, and decreased during the subsequent suckling and weaning periods. As in the liver, C/EBP
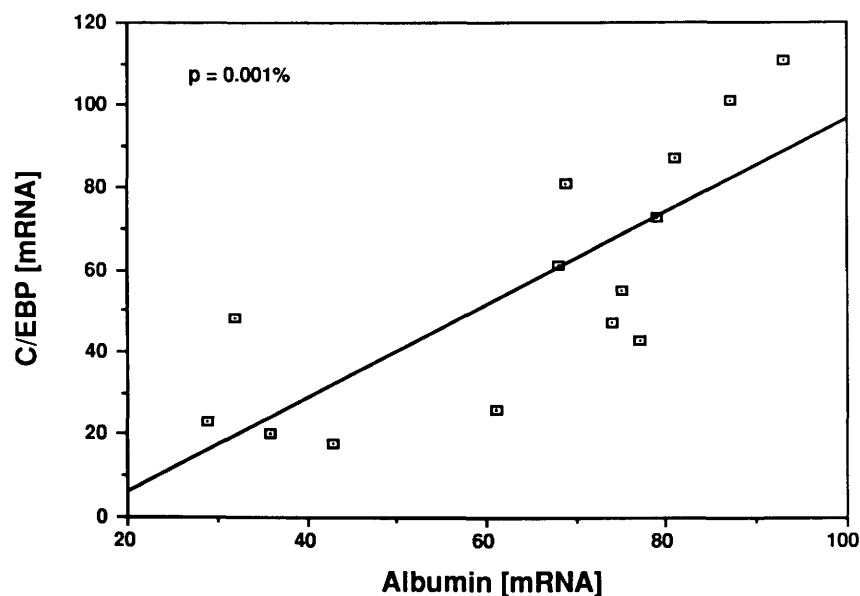
Figure 5. Correlation of C/EBP and albumin mRNA levels in adult mouse liver. Total cellular RNA was isolated from individual adult male BALB/cByJ mice that were killed at 4-hr intervals over a 3-day period. The RNA was used to prepare slot blots, which subsequently were hybridized to radioactive probes specific to either the rat C/EBP gene or the rat albumin gene. The amount of total RNA remaining in each slot after hybridization was measured by UV shadowing the nylon filter. The amount of each mRNA present in each sample was determined by quantitative laser densitometry of an autoradiogram. The graph shows the amount of C/EBP mRNA plotted as a function of the amount of albumin mRNA present per microgram of total RNA in each mouse liver. Spearman's rank correlation coefficient was used as a statistical test for measuring the probability of correlation between the two variables.

A Rat Liver Development C/EBP

C Mouse Liver Development C/EBP

B Rat Intestinal Development C/EBP

D Mouse Intestinal Development C/EBP

Figure 6. Developmental regulation of C/EBP mRNA accumulation in small intestine and liver of rats and mice. For each tissue, total cellular RNA was isolated from fetal, neonatal, and adult animals, as described in Materials and methods. Dot blots were hybridized to a ^{32}P-labeled C/EBP probe, and autoradiograms were analyzed by quantitative laser densitometry. For each of the four types of RNA collected, the highest level of C/EBP mRNA observed during the various stages of development was arbitrarily set at 100. Day 1 of gestation was designated as the time when a vaginal plug was observed in the mothers. Day 1 of postnatal life was designated as the day of birth (identified by the letter B and an arrowhead on each graph). The time points to the *left* of the arrow are fetal, whereas those to the *right* are postnatal. (A and B) The developmental regulation of C/EBP mRNA in rat liver and rat small intestine, respectively; (C and D) the developmental regulation of C/EBP in mouse liver and mouse small intestine, respectively.

mRNA concentration was observed to rise two- to threefold at the transition from the end of the weaning period to young adulthood. The similarity of developmental patterns of C/EBP mRNA accumulation in liver and intestine raises the possibility that expression of this gene may be modulated by common mechanisms in both tissues.

*Expression of C/EBP mRNA and protein during adipocyte differentiation*

The preliminary analyses of C/EBP mRNA accumulation outlined in the preceding sections have revealed a nonrandom tissue distribution. Furthermore, fluctuations relating to developmental stage and, perhaps, physiologic state were observed also. The complexity of these in vivo data underscores the potential value of a cell culture system capable of recapitulating C/EBP regulation. Such a system might facilitate a systematic study of the factors that modulate C/EBP expression. Having observed high levels of C/EBP mRNA in fat tissue from rats and mice, we examined protein and mRNA expression as a function of differentiation of cultured preadipocyte cells.

Cultured 3T3-F442A cells were grown to confluency, and either kept in growth medium (GM) or switched to a

culture medium that facilitated differentiation (Materials and methods). Total RNA was extracted at regularly spaced intervals after exposure to differentiation medium (DM), electrophoresed on a denaturing agarose gel, and stained with ethidium bromide to evaluate the intactness of the RNA, which was then transferred onto a nylon filter. The filter was exposed successively to several radioactive, recombinant DNA probes specific to three different mRNAs. As shown in Figure 7 (top), mRNA encoding glycerol-3-phosphate dehydrogenase (GPDH), an enzyme marker diagnostic of terminally differentiated adipocytes (Dobson et al. 1987), first was observed 6 days after exposure to DM and increased at the 8- and 11-day time points. After obtaining an autoradiographic exposure of the filter, it was washed under conditions that removed the radioactive GPDH probe and was subjected to hybridization with a probe specific for α-tubulin (Fig. 7, middle). The fact that α-tubulin mRNA concentration did not change markedly during the interval of GPDH induction served as a control to demonstrate both the specificity of the differentiation process and the intactness of the various RNA samples. Finally, the filter was washed under conditions that removed ~75% of the hybridized α-tubulin probe and exposed to a radioactive probe specific to C/EBP mRNA. As shown in Figure 7 (bottom), C/EBP mRNA was barely detectable 4 days postexposure to DM and increased markedly in concentration at the 6-, 8-, and 11-day time points. The time of initial appearance of C/EBP mRNA, 4–6 days postinduction, corresponded with the time at which the first foci of lipid-containing cells were noted. Moreover, the induction of C/EBP mRNA was completely dependent on exposure to DM; confluent cells maintained in GM for 8 days failed to express C/EBP and failed to show morphological evidence of differentiation. Finally, comparable patterns of C/EBP mRNA accumulation were also observed during the course of differentiation of 3T3-L1 cells from preadipocytes to adipocytes (data not shown).

To test whether the pattern of appearance of C/EBP mRNA in differentiating adipocytes correlated with the appearance of C/EBP protein, extracts of 3T3-L1 cells were prepared at timed intervals following exposure to DM, electrophoresed on denaturing polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies specific to C/EBP. Figure 8 (top) shows the profile of proteins present at different stages during the differentiation of 3T3-L1 cells, as diagnosed by Coomassie Blue staining. The three panels shown below the photograph of the stained gel represent autoradiographic exposures of Western blots conducted to examine the temporal appearance of C/EBP protein. After electrophoretic transfer onto a nitrocellulose filter, the proteins were probed using a mixture of two rabbit antibodies, one specific to an epitope located immediately amino-terminal to the DNA-binding domain of C/EBP (Landschulz et al. 1988b) and another specific to an epitope located within the carboxy-terminal 15 amino acids of C/EBP (W. Landschulz and S. McKnight, unpubl.). As shown in the top Western blot assay, this mixture of antibodies reacted
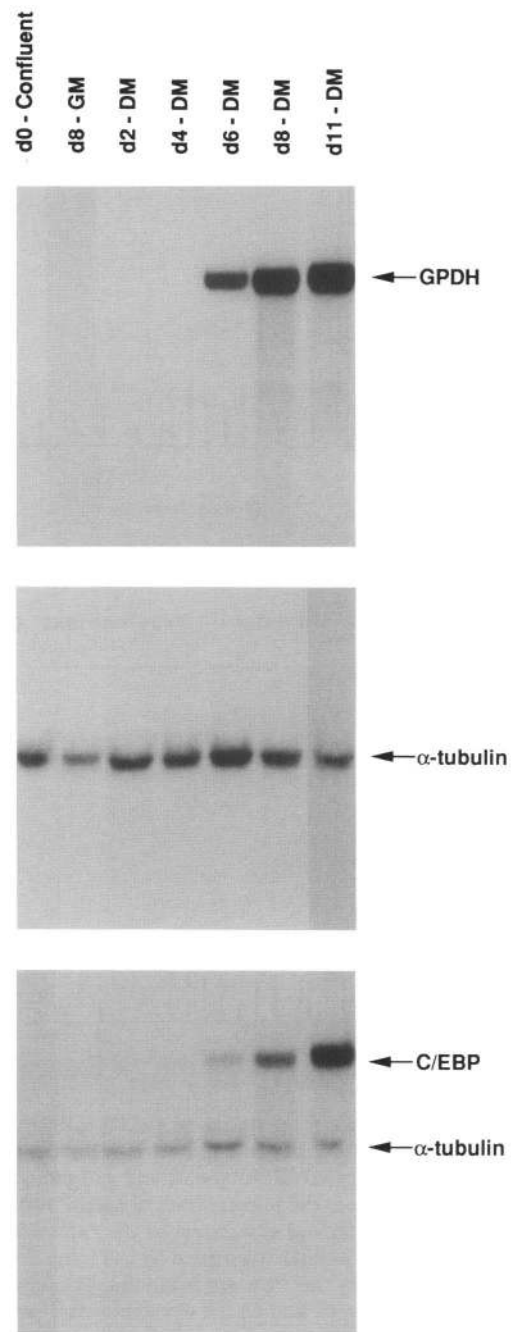
Figure 7. Developmental regulation of C/EBP mRNA accumulation in cultured adipocytes. 3T3-F442A preadipocytes were cultured in GM until confluent (Materials and methods). Starting on day 0, cells were either maintained as preadipocytes in GM or induced to differentiate into mature adipocytes by exposure to differentiation medium (DM). Six days after exposure to DM, groups of cells began to accumulate lipid. Total cellular RNA was isolated on days 0, 2, 4, 6, 8, and 11 of treatment with DM. A Northern blot was prepared using 20 μg of each RNA sample. Autoradiograms of this blot are shown, which sequentially were hybridized to ^{32}P-labeled probes specific to mRNAs encoding GDPH (*top*), α-tubulin (*middle*), and C/EBP (*bottom*). Note that the second lane (labeled *d8-GM*) contains RNA from confluent preadipocytes that were maintained 8 days on GM alone.
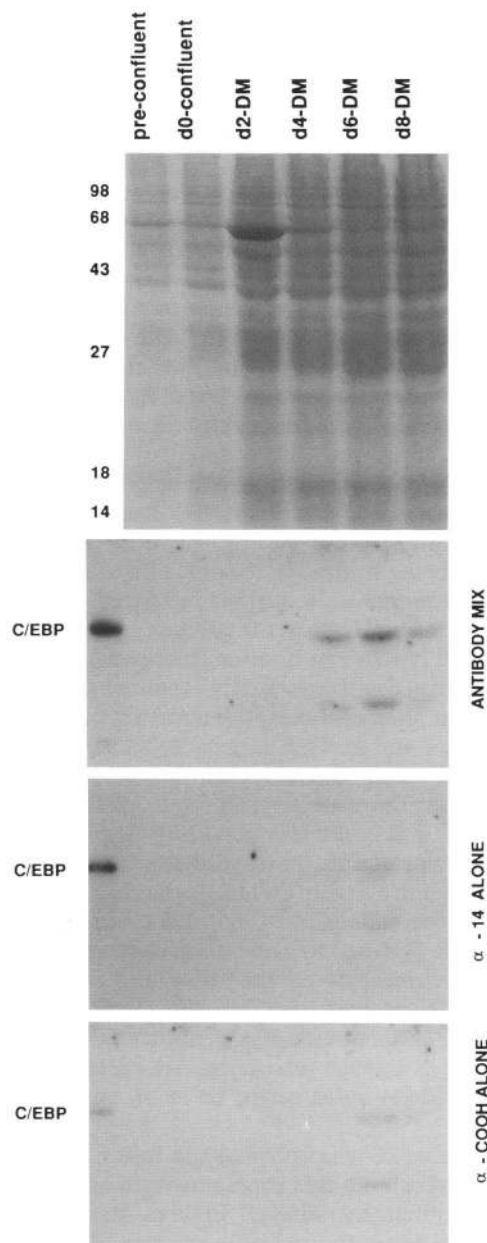
Figure 8. Developmental regulation of C/EBP mRNA accumulation in cultured adipocytes. 3T3-L1 cells were induced to differentiate and examined by Western blot analysis, as described in Materials and methods. (*Top*) A Coomassie Blue-stained SDS–polyacrylamide gel of total cellular proteins present as a function of the time course of differentiation. The sizes of molecular weight markers are noted at *top left*. The three *bottom* panels show Western blot analyses of electrophoresis gels that were run in parallel with that of the *top* panel. A bacterial extract expressing full-length C/EBP was added as a positive control in the *left-most* lane of each Western blot. In the second panel from the *top*, a mixture of antibodies specific for an internal peptide and a carboxy-terminal peptide of C/EBP was used to detect C/EBP expression. Full-length C/EBP and a 30-kD fragment first appear after 4 days of culture in DM, and reach peak levels at the 6-day time point. (*Bottom*) Western blot reactivity with separate antiserum preparations are shown. Each antiserum stains both the 42- and 30-kD polypeptide species.

with two polypeptide species whose expression profiles correlated with the initial appearance of lipid-containing cells (6 days post-induction; data not shown). One of these polypeptide species corresponded in size to that of intact C/EBP (42kD). The other corresponded with that of a smaller polypeptide that migrated with an apparent molecular mass of ~30kD. A similarly truncated form of C/EBP has been observed in rat liver nuclei (Landschulz et al. 1988b).

The nature of the two C/EBP-like polypeptides was evaluated in more detail using Western blots probed separately with the two antibody reagents (α-14 and α-COOH). As shown in the two lower panels of Figure 8, both antibody reagents reacted with both polypeptide species. These observations are consistent with the identification of the 42- and 30-kD species as bona fide products of the C/EBP gene. They suggest further that if generated by proteolysis, the 30-kD form maintains an intact DNA-binding domain [because the two epitopes flank the C/EBP DNA-binding domain (see Landschulz et al. 1988b)]. We are unaware of the significance of the 30-kD form of C/EBP.

The results of Western blot analysis of C/EBP expression in differentiating 3T3-L1 cells did not perfectly match that of C/EBP mRNA expression in differentiating 3T3-F442A cells. Although C/EBP mRNA and protein were observed to be induced with similar kinetics in the two adipocyte cell cultures, C/EBP mRNA continued to increase in concentration in 3T3-F422A cells at a time when C/EBP protein concentration was tapering off in 3T3-L1 cells. Given that the experiment was carried out with different adipocyte cell cultures, we do not know whether this discrepancy is significant.

Discussion

This paper presents the results of experiments that provide new and potentially useful information about the gene that encodes C/EBP and its pattern of expression in rodents. *Cebp* is single copy in the mouse genome and maps at a position very close to the structural gene encoding glucose phosphate isomerase on chromosome 7. The single-copy nature of the gene encoding C/EBP, coupled with its intron-free architecture (Landschulz et al. 1988b), facilitated direct hybridization assays of mRNA distribution as a function of tissue type and developmental stage.

Prior to this study, virtually nothing was known about the pattern of cell and tissue types that would use C/EBP. Our initial expectation was that the protein might be present ubiquitously. This bias was influenced by the fact that C/EBP-binding sites (enhancer core sequences and CCAAT homologies) are common to mRNA-coding genes expressed in a wide variety of cell types. However, other observations raised the distinctly different possibility that C/EBP might be restricted to a single cell type. Although identified initially in the McKnight laboratory as a prominent, heat-stable DNA-binding activity in rat liver nuclear extracts (Graves et al. 1986; Johnson et al. 1987), C/EBP-like activities had

not been observed in analogous studies that used other sources of nuclear protein (e.g., Lee et al. 1987; Mitchell et al. 1987). In other words, it was possible that C/EBP was a liver-specific DNA-binding protein. The results presented herein point to an interpretation intermediate to the aforementioned extremes. C/EBP mRNA is present in certain adult tissue types and absent or present at very low levels in others.

One way to begin interpreting the tissue distribution of C/EBP is to consider the physiologic or ontogenic similarities shared by tissues that express the highest levels of C/EBP mRNA. Liver, gut, lung, adipose tissue, adrenal gland, and placenta are derived from different germ layers and are not linked by any obvious physiologic circuitry. On the other hand, adipocytes are highly specialized in that they are the major depot for lipid stores in times of nutritional abundance. Perhaps the capacity of the other tissues to metabolize lipids at high rates is related to the fact that they also express significant levels of C/EBP mRNA. Parenchymal cells of the liver produce large quantities of lipids, lipoproteins, lipid carrier proteins (such as albumin), and fatty acid-binding proteins. Differentiated endothelial cells of the gut use fatty acid-binding proteins to sequester fat from the intestinal lumen, undertake the initial chemical modification of diet fat, and produce lipoproteins to transport lipids to other organs. Although the lung is not involved with nutritional balance, it produces unusually large quantities of lipids and associated proteins that constitute the surfactant lining of alveolar sacks. Finally, in addition to its vital role in fetal nutrition, the placenta produces large quantities of cholesterol-derived sterols. Perhaps, the C/EBP mRNA present in the adrenal gland of adult rats is related to the fact that this organ also produces large amounts of steroids.

We speculate that C/EBP may modulate the transcription of genes that are expressed in tissues where synthesis or metabolism of lipids is an important part of their normal physiology. The enzyme GPDH might represent just such a target. Although expressed ubiquitously, GPDH levels are particularly high in differentiated adipocytes. Beyond the general concordance of C/EBP and GPDH expression in adult white and brown adipose tissue, we make note of their similar profiles of induction during the differentiation program of cultured adipocytes (Fig. 7).

Although C/EBP might facilitate the coordinated expression of a set of proteins commonly required by a number of different tissue types, we can offer at least one reason to believe that its gene regulatory effects will be more subtle than the scheme outlined in the preceding paragraph. Evidence obtained from a number of different studies has indicated that C/EBP is required for high rates of albumin mRNA synthesis. Whether synthesized in bacterial cells or purified from rat liver nuclei, C/EBP protein binds to the albumin promoter (P. Johnson, S. Cereghini, and M. Yaniv, unpubl.). The relevance of such observations is supported by in vitro transcription studies of the albumin promoter that demonstrated the involvement of a protein exhibiting properties similar to C/EBP (Lichtsteiner et al. 1987), as well as

by mutagenesis studies that have shown the C/EBP-binding site to be crucial for albumin promoter function (Gorski et al. 1986; Heard et al. 1987; Lichtsteiner et al. 1987). Now we have observed that albumin mRNA levels vary in adult mouse liver in a manner that correlates with that of C/EBP mRNA (Fig. 6). Finally, and perhaps most convincingly, transient transfection studies using a C/EBP expression vector have provided evidence that C/EBP is potent *trans*-activator of the albumin gene (A. Friedman, W. Landschulz, and S. McKnight, in press). If we are correct in assuming that C/EBP is an activator of albumin gene expression in the liver and that it is expressed in other tissues that do not synthesize albumin mRNA (Figs. 2 and 3), then its simple presence would appear to be insufficient to ensure gene activation. More detailed experiments pertaining to this interpretation will be presented in a subsequent report (A. Freidman, W. Landschulz, and S. McKnight, in press).

Beyond being limited in its tissue distribution, C/EBP expression seems to be further restricted to terminally differentiated cells. Only the parenchymal cells of adult liver tested positive in immunofluorescence assays (Fig. 4), and cultured adipocytes only produced C/EBP mRNA and protein after assuming the terminally differentiated state (Figs. 7 and 8). This pattern of C/EBP expression is quite unlike that of the nuclear oncogenes whose protein products share amino acid sequence similarity with the DNA-binding domain of C/EBP (Landschulz et al. 1988a,b). For example, MYC protein expression tends to be far higher in actively dividing cells than in terminally differentiated cells (Cole 1986). Likewise, the mRNAs that encode FOS and JUN are more concentrated in cells that are cycling acvtively than those that are mitotically quiescent (Greenberg and Ziff 1984; Ryder et al. 1988). Because C/EBP expression is reciprocally related to the expression of nuclear oncogenes, we speculate that one of its regulatory roles might be as an antiproliferative agent.

Irrespective of what physiologic role C/EBP actually plays, the results in this paper provide a basis for testing several explicit hypotheses. In vivo studies can now focus on altering the physiologic state of mice via dietary, hormonal, or genetic manipulation to observe alterations in C/EBP expression in certain target tissues such as liver and adipose. In addition, our results suggest that use of preadipocyte cell lines may provide an important in vitro system to assess C/EBP function. Finally, it should now be possible to determine whether aberrant C/EBP expression in proliferative cells results in an antiproliferative effect.

Materials and methods

Animals

Timed pregnant, suckling, and adult male Sprague–Dawley rats were obtained from Sasco (St. Louis, Missouri). BALB/cByJ mice were obtained from the production facilities of the Jackson Laboratory. All animals were maintained in rooms with a 12-hr light cycle and were fed standard chow diets. Suckling pups were maintained with their mothers until time of sacrifice. Rats and suckling mice were killed by decapitation. Adult mice were killed by cervical dislocation.

Preparation of tissue RNA

Organs were removed from individual animals and frozen immediately in liquid nitrogen. Total cellular RNA was extracted using the guanidine isothiocyanate method (Chirgwin et al. 1979). For the adult mouse tissue distribution studies, tissues were pooled from five 10-week-old male BALB/cByJ mice. For the adult rat tissue distribution studies, tissues were pooled from ten 250-gram adult males that were derived originally from two litters. For the developmental studies in rat liver and small intestine, tissues were pooled from 10–30 animals per time point. For studies of gene expression in livers from individual adult mice, fourteen 8-week-old male BALB/cByJ mice were housed in separate cages in one mouse room for a 3-day adaptation period. Single mice were sacrificed every 4 hr. Livers were dissected, quick-frozen in liquid nitrogen, and stored at −80°C for subsequent preparation of total RNA.

RNA dot-blot hybridization

Dot blots of rat RNA were prepared and hybridized as described by Sweetser et al. (1988). Briefly, four amounts (0.5, 1, 2, and 3 μg) of each sample were applied to nitrocellulose filters with a template manifold. Yeast tRNA was added to the samples so that a total of 3 μg of RNA was applied to each template slot. Dot blots of mouse RNA were prepared in similar fashion using Zetabind (AMF, Meriden, Connecticut) nylon filters instead of nitrocellulose. In some cases, the yeast tRNA was not included so that the filters could be UV-illuminated to measure RNA retention following hybridization. Nylon filters were hybridized as described elsewhere (Heuckeroth et al. 1987). The relative abundance of a specific mRNA was determined by quantitative scanning laser densitometry of filter autoradiographs using a Hewlett–Packard 3392A integrator and a Bio-Rad model 620 video densitometer. Following hybridization and autoradiography, filters were UV-illuminated to determine the amounts of total RNA remaining fixed to the filter (Thurston et al. 1989). The filter was placed RNA side down on a 254-nm UV light box. A photographic negative then was obtained using a Kodak Wratten 15 filter and Polaroid type 55 film. The amount of RNA present in each dot then was determined by quantitative scanning laser densitometry of the film negative, as described above.

Chromosome mapping

DNA from spleens of inbred and recombinant inbred mouse stains was purchased from the DNA Resource of the Jackson Laboratory. Mapping of the *Cebp* gene was carried out using RFLPs, as described previously (Heuckeroth et al. 1987). Briefly, DNA samples were digested with restriction enzymes, electrophoresed through 0.8% agarose gels, transferred to Zetabind nylon filters, and hybridized to a radiolabeled C/EBP probe. The RFLPs detected in the various strains were used to identify the three *Cebp* alleles and to determine the chromosomal location of the gene, as described in Table 1.

Northern blot of mouse tissue RNA

Ten micrograms of each RNA sample was electrophoresed through a 1.0% agarose gel containing 7% formaldehyde, transferred to a Zetabind nylon filter, and hybridized to a radiolabeled DNA probe, as described previously (Birkenmeier et al. 1989). After hybridization and autoradiography, the nylon filter was UV-illuminated to locate the positions of the 18S and 28S rRNAs.

Radiolabeled probes

DNA probes were radiolabeled using the random hexamer method (Feinberg and Vogelstein 1983). The C/EBP DNA probe corresponded to a 1.5-kb rat cDNA fragment, covering slightly more than half of the 2.7-kb C/EBP mRNA (Landschulz et al. 1988b). The albumin DNA probe corresponded to a partial rat cDNA clone, termed pRSA 13 (Sargent et al. 1981). The α-tubulin DNA probe corresponded to a 1.0-kb human cDNA clone, kindly provided by A.J. Lusis (University of California, Los Angeles). The mouse GDPH DNA probe corresponded to a 0.8-kb genomic *Hin*dIII fragment (Ireland et al. 1986).

C/EBP mRNA expression in 3T3-F442 cells

Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20 mM glutamine and 4500 mg/ml of glucose, and lacking sodium pyruvate. GM was supplemented with 10% calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. DM was supplemented with 10% fetal bovine serum, 5 μg/ml of insulin, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were split 1 : 5 and maintained in GM. When they became confluent (day 0), they were induced to differentiate by addition of DM. Confluent cells that were kept in GM failed to differentiate and were used as controls. Total cellular RNA was isolated at various time points. RNA samples (20 μg) were electrophoresed through a 1.25% agarose gel containing 7% formaldehyde. The gel then was blotted onto Zetabind nylon filters and hybridized to a radiolabeled C/EBP probe, as described previously (Jacobsson et al. 1985).

C/EBP protein expression in 3T3-L1 cells

3T3-L1 cells (a generous gift from M. Daniel Lane) were induced to differentiate according to published procedures (Student et al. 1980). The cells were grown to confluency and left for 2 days in GM. Adipogenesis was induced by a 2-day treatment with 390 ng/ml of dexamethasone and 115 μg/ml of methylisobutylxanthine in DM. The medium was replaced every 2 days with fresh DM. Cell proteins were harvested during the course of induction by removing the medium, washing with PBS and lysing in 3 ml of 0.1% SDS, 5 mM EDTA, 10 mM Tris (pH 7.5), and 100 mM NaCl. Cell lysates were scraped into a 15-ml polypropylene tube, sonicated for 30 sec, and treated with 4 volumes acetone at −20°C. Precipitated proteins were collected by centrifugation, resuspended in SDS–sample buffer, and separated by SDS–polyacrylamide gel electrophoresis (Laemmli 1970). After electrophoretic transfer to nitrocellulose, C/EBP was detected using antibodies specific to either an internal 14-amino-acid peptide (Landschulz et al. 1988b) or a peptide corresponding to the carboxy-terminal 15 amino acids of C/EBP (W. Landschulz and S. McKnight, unpubl.), or a combination of the two antibodies. Antibody–antigen complexes were visualized by autoradiography after staining with ^{125}I-labeled protein A.

Immunofluorescent staining of liver tissue

Liver tissue was dissected from adult mice and deposited onto subbed slides by a method developed by J. Gall. Roughly 1-mm³ pieces of liver tissue were placed in 100 μl of OR2 buffer [82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 5 mM HEPES (7.8)]; (Wallace et al. 1973). The tissue was teased apart and squashed onto the slide using a siliconized coverslip. The slide then was submerged in liquid nitrogen for 30 sec and removed, and the coverslip was popped off with a razor blade. Then the tissue was fixed in 95% ethanol overnight. Before staining, slides were exposed to 3 M urea for 5 min

at 24°C (Hausen and Dryer 1982). Antibody staining was carried out after a 30-min blocking reaction using 10% horse serum (HS) in Tris-buffered saline [TBS: 10 mM Tris (pH 7.5), 150 mM NaCl]. Squashed tissue was incubated at room temperature for 1 hr with anti-C/EBP antibodies (Landschulz et al. 1988b) diluted 1 : 100 into 2.5% HS in TBS. Slides were washed three times with 2.5% HS in TBS and then incubated for 1 hr with rhodamine-labeled anti-rabbit IgG antibodies diluted into 2.5% HS in TBS. After washing unbound antibody away, a coverslip was mounted with a drop of 50% glycerol in TBS containing 0.2 μg/ml of DAPI. Control reactions carried out using either preimmune serum or second antibody alone showed no evidence of hepatocyte-specific nuclear staining.

Acknowledgments

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