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Cell-specific transcriptional control of the mouse DNA-binding protein mC/EBP

(cell specificity/transcriptional regulation)

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ABSTRACT **The mRNA encoding the mouse homolog of C/EBP, a rat DNA-binding protein that participates in activating a number of genes in hepatocytes, is present in liver cells at a far higher concentration than in most other cells, including spleen, kidney, muscle, and the majority of the brain. However, fat cells and intestinal cells contain 25–50% as much mRNA as liver cells. "Run-on" experiments show that the basis for the restricted cellular distribution of the mouse C/EBP mRNA is transcriptional regulation of the gene. We also show that disruption of cell–cell contacts incident to liver cell dispersion results in a prompt and extensive reduction in mouse C/EBP transcription as we had earlier shown to be the case for a group of 10 genes transcribed in a hepatocyte-specific fashion. In contrast, breaking cell contacts and plating the hepatocytes in culture leads to a prolonged increase in transcription of the Jun-B gene that encodes a widely distributed transcription factor. These results illustrate that the regulation of expression of a mammalian regulatory protein with limited tissue distribution is controlled at the level of transcription and depends on cell contacts.**

We have previously shown that active transcription in hepatocyte nuclei, but not in brain, kidney, spleen, or cultured cell nuclei, was the basis for the presence in liver of an array of "liver-specific" mRNAs (1, 2). We and many other laboratories have been engaged in identifying regulatory regions of the genes encoding these transcriptionally controlled mRNAs in order to study the proteins that bind to such regulatory regions (3–8). An important unanswered question about proteins that regulate hepatocyte-specific transcription is whether any such regulatory molecules will be limited in their cellular distribution perhaps only to hepatocytes. Alternatively, the important regulatory DNA-binding proteins may be present in all cells but active only in the liver because of a hepatocyte-specific modification.

In the past year it has become clear that extracts of hepatocytes, but not of several other cell types, contain at least four proteins (9) that can interact with DNA regions required for maximal transcription of transthyretin (TTR), α_1-antitrypsin (α1-AT), α- and β-fibrinogen, albumin, and other genes expressed mainly in hepatocytes (4–8). In addition, an alternative spliced mRNA encoding an NF-1-like DNA-binding protein, has a higher concentration in the liver than elsewhere (10). Not all of these liver-enriched proteins bind to every gene expressed in hepatocytes, but in all cases the identified proteins bind to sites present in at least two different genes. Moreover, all of the tested genes so far require two or more proteins for maximal transcriptional activity. C/EBP, an enhancer-binding protein purified from rat liver by virtue of its affinity to viral enhancers (11), is one of these proteins (12). We mapped six sites in the TTR and α1-AT enhancer and promoter regions that bound the same protein in liver extracts that was absent in brain, spleen, or kidney extracts (4, 9, 13). In an artificial construct, multiple binding sites from the TTR gene can act as an enhancer for RNA synthesis from a reporter gene, indicating that the relevant protein is very likely a positive-acting transcription factor (13). The liver protein could be highly purified by DNA-affinity chromatography using a TTR binding site (12). Finally, these same sites in TTR and α1-AT genes were also shown to bind C/EBP obtained as a fusion protein produced in bacteria from the cloned C/EBP cDNA (12, 14). From these results it appeared to us that C/EBP was not widely distributed but was a transcription factor that is greatly enriched in liver compared with other cell types.

Here we report the isolation of the mouse homolog to C/EBP (mC/EBP), document a limited tissue distribution of the mRNA as judged by RNA blot hybridization (Northern blots) and *in situ* experiments, and show that differential regulation of transcription underlies the difference in distribution of the mRNA. Finally, we show that regulation of mC/EBP transcription, like that of other hepatocyte-expressed mRNAs, depends on cell contact.

MATERIALS AND METHODS

Gene Cloning and Sequencing. The gene encoding mC/EBP was selected by screening a phage λgt11 mouse liver cDNA library (prepared by E. Paulson) by using the rat cDNA insert (provided by W. H. Landschulz and S. L. McKnight) of the gene as probe (14). Approximately 2.5×10^5 recombinant phages were screened by plaque hybridization under low-stringency conditions. Nitrocellulose filters were prehybridized in 35% formamide/5 × SSPE/0.1% sodium dodecyl sulfate (SDS)/3× Denhardt's solution/100 μg of denatured salmon sperm DNA per ml at 42°C for 2–3 hr (1× SSPE = 150 mM NaCl/10 mM $NaH_2PO_4 \cdot H_2O$/1 mM Na_2EDTA; Denhardt's solution = 0.02% bovine serum albumin/0.02% pyrrolidone/0.02% Ficoll). Hybridization was carried out overnight in the same solution with the addition of probe ($\approx 10^6$ cpm/ml of hybridization solution). Washes were performed twice in 0.30 M NaCl/0.03 M sodium citrate, pH 7, containing 0.1% SDS at 58°C for 30 min. Positive clones were selected and purified after three rounds of hybridization, and the largest insert of the three overlapping positive clones was subcloned to a pGem-2 plasmid vector for further analysis. Different fragments of this cDNA insert were subcloned into the pGem-1, pGem-2, or pBluescript vectors and were sequenced by the chain-elongation method (15).

Northern Blot Analysis and RNA *in Situ* Hybridizations. Total RNA was isolated from various tissues as described

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Abbreviations: α1-AT, α_1-antitrypsin; TTR, transthyretin; C/EBP, rat enhancer-binding protein; mC/EBP, mouse homolog of rat C/EBP.

(16). Northern blot analysis of total RNA (25 μg per lane) was carried out by electrophoresis in 1% formaldehyde/agarose gel, transfer to nitrocellulose, and hybridization to appropriate DNA fragments labeled to a specific activity of 10^8 cpm/μg by random priming (17) under high-stringency conditions. Prehybridization, hybridization, and washing of the nitrocellulose filters were performed by standard methods (18). *In situ* hybridizations were performed as described (19) with an antisense ^{35}S-labeled RNA prepared by *in vitro* transcription of an 800-base-pair (bp) mC/EBP cDNA fragment.

Isolation of Mouse Nuclei from Various Tissues and Nuclear Run-On Assays. Nuclei from different mouse tissues were isolated, and nuclear run-on assays were performed as described by hybridizing labeled nuclear RNA (1, 2, 20, 21) to an excess of different cDNAs immobilized (6 μg per sample) on nitrocellulose filters and subjecting washed and RNase-treated filters to autoradiography (−70°C for 1–3 days with intensifying screens). Quantitation of the autoradiographic signal was carried out by densitometry (20).

RESULTS

Cloning of mC/EBP cDNA. The C/EBP gene from rats was recently cloned (14). With a sample of this clone (kindly provided by S. L. McKnight), we selected the mouse counterpart (mC/EBP) to the rat gene from a λgt11 mouse liver cDNA library. As was reported for rat liver (14), the mRNA was fairly scarce (3 clones out of 2.5×10^5 phage plaques). Partial sequence analysis of the cDNA insert of this clone confirmed an open reading frame with almost complete amino acid sequence identity and 90–95% nucleic acid identity with the rat C/EBP in the protein coding region. (In the 159 amino acids encoded by the sequence that we have analyzed, a leucine replaces phenylalanine at position 82 of the rat sequence, an alanine at position 98 is deleted, and a leucine replaces methionine at position 237.) The gene encoding mC/EBP is about 2.7 kilobases (kb) long, single copy, and intronless (K.G.X. and J.E.D., Jr., unpublished results). In addition, by analysis of restriction fragment length polymorphisms in various strains of mice, we have assigned the gene to mouse chromosome 7 (22).

Tissue Distribution of the mC/EBP mRNA. The relative steady-state concentrations of mC/EBP mRNA were determined by Northern blot analysis of total RNA from mouse brain, fat, kidney, liver, heart, and intestine tissues. Fat tissue was included because S. L. McKnight informed us that this tissue in rats contained C/EBP. The liver contained at least 50-fold more mC/EBP mRNA than did brain, spleen, or kidney tissue (Fig. 1*A*). Long exposures of the film revealed faint bands in the kidney, heart, and spleen samples. Fat tissue contained about 50%, and small intestinal tissue contained about 25% as much mC/EBP mRNA as did the liver sample. A single mRNA band of similar size (≈2.7 kb) was detected in all of these RNA samples. In the Northern experiments, the same amount of total RNA judged by UV absorption was added in each lane. After hybridization to mC/EBP, the filters were stripped and rehybridized with probes to mouse β-actin (top band of controls) and a mouse variant of a rat proteoglycan (bottom band of controls); the probe was selected by M. Salditt-Georgieff from a mouse liver cDNA library by using a rat proteoglycan clone kindly provided by E. Ruoslahti. These two mRNAs were present in all tissues, although the concentrations obviously varied. Another test of the distribution of expression of mC/EBP mRNA in various cells is *in situ* hybridization. Fig. 1*B* shows one such example of the limited cellular distribution of mC/EBP mRNA in mouse brain. Although the level of this mRNA in total brain was very low, some cells in this midbrain section have a large concentration of the mC/EBP mRNA. In

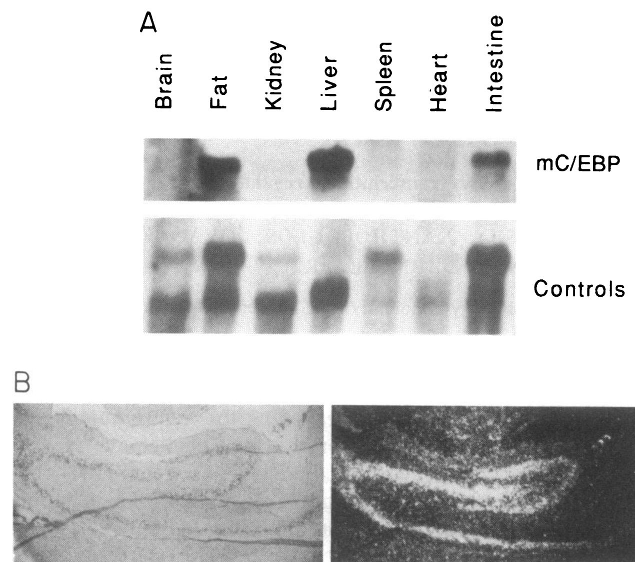

FIG. 1. Distribution of mC/EBP mRNA in various tissues. (*A*) Northern blot analysis of total RNA (25 μg per lane) from various tissues was carried out by electrophoresis, transfer to nitrocellulose, and hybridization to an 800-bp center fragment of mC/EBP (see Fig. 2*B*). Autoradiographic exposure (with screens) was carried out for 36 hr. Control blots were performed by washing the filter twice in 0.015 M NaCl/0.0015 M sodium citrate, pH 7 containing 0.1% SDS at 90°C for 20 min to remove the labeled mC/EBP probe and rehybridizing with a labeled mouse actin probe (top control band) and a mouse clone homologous to a rat proteoglycan cDNA insert (bottom control band). Exposure time was 18 hr. (*B*) A transverse brain section at the level of the hippocampus was treated for *in situ* hybridization (19) and exposed to labeled antisense RNA complementary to mC/EBP mRNA. The strip of hybridizing cells is seen as a black streak in the light-field photograph (*Left*) and as white dots in the dark-field photograph (*Right*). Exposure time was 1 week.

contrast, mC/EBP mRNA is uniformly distributed in all hepatocytes (a full description of mC/EBP mRNA in various brain cells and in developing embryos will be reported). From these results it appeared that the mC/EBP mRNA is definitely not present in all cells but is present in hepatocytes, fat cells, intestinal cells, and a minority of brain cells have a high concentration of this mRNA.

Nuclear Run-On Transcription Analysis. Nascent chain analysis of nuclear RNA synthesis, the so-called run-on transcription analysis, was used to determine the basis for the differential tissue distribution of mC/EBP mRNA. We first needed to determine whether transcription of a gene that yields rare mRNAs gave clear signals in these assays. Liver and kidney nuclei were used to prepare labeled nascent nuclear RNA (1, 20, 21), and samples were hybridized to several cDNA clones, including albumin and mC/EBP cDNA (Fig. 2*A*). The mC/EBP transcriptional signal in the liver nuclei was very strong, ≈30% of that for albumin (the probe size was 2.2 kb for mC/EBP and 0.8 kb for albumin). In contrast, the kidney signal was barely above the background and was less than 5% that of the liver. The autoradiograms were scanned by densitometer and normalized to the $tRNA^{Met}$ signal, which was comparable in all samples. Since albumin mRNA represents about 1–2% of total liver mRNA (21) but has a transcriptional signal only about 2–3 times that of mC/EBP, whose mRNA is quite scarce, the mC/EBP transcript must have a relatively short half-life.

That the liver hybridization signal for mC/EBP was not a spurious one (for example, from a repetitive transcribed sequence) was indicated first by the fact that labeled kidney nuclear RNA gave little or no signal above background. In addition, genomic Southern blots using several restriction enzymes and mC/EBP cDNA as labeled probe showed

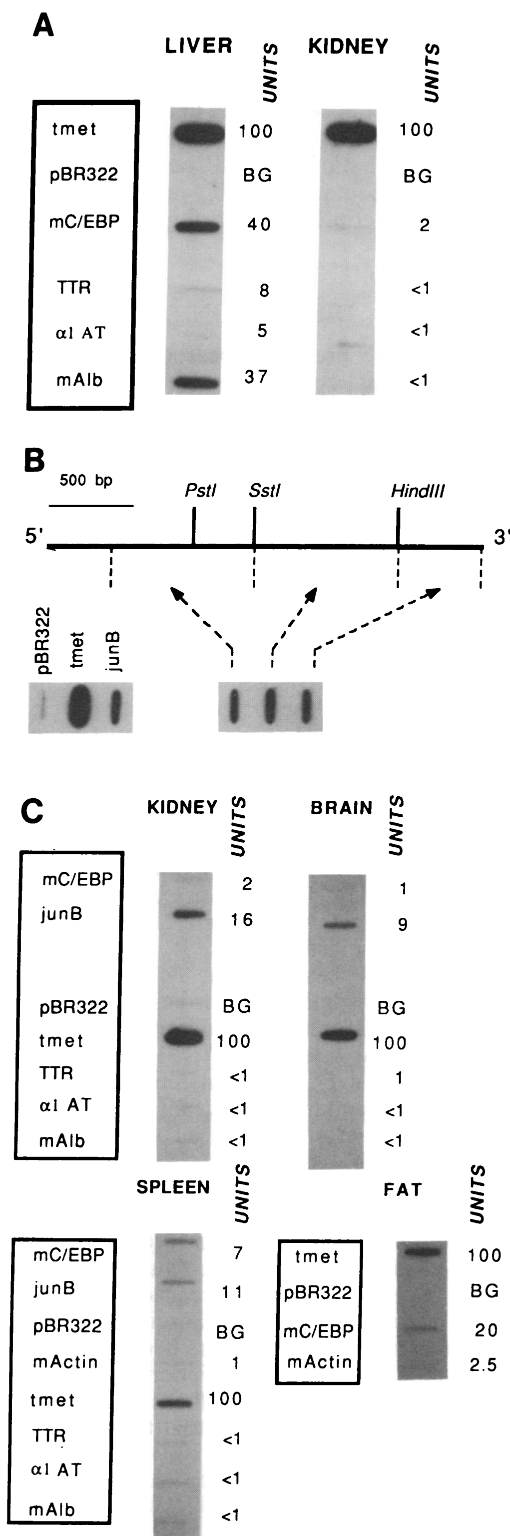

FIG. 2. Transcriptional analysis of mC/EBP in various mouse tissues. Run-on analysis including labeled nuclear RNA preparation, hybridization, and autoradiography was as described (this paper and refs. 1, 2, 20, and 21). Densitometric tracings of preflashed x-ray films with appropriate exposures were made to estimate relative intensities. The results presented as arbitrary units were obtained by normalizing each signal to the $tRNA^{Met}$ (tmet) signal as 100 in each case after subtraction of the background (BG). The DNA samples used were $tRNA^{Met}$ (tmet), Gemini plasmid 1 (pGEM), pBR322, a 0.4-kb cDNA insert of mouse α1-AT (13), a 0.6-kb cDNA insert of TTR (3), a 2.2-kb cDNA insert of mC/EBP, a 0.8-kb cDNA insert of mouse albumin (mAlb; a gift of S. Tilghman), a 1.8-kb cDNA insert of *Jun-B* (a gift of D. Nathans; see ref. 23), and a 0.8-kb cDNA insert of mouse β-actin (mActin; a gift of D. Grayson). (*A*) Input of 5×10^6 cpm of labeled nascent RNA from liver and 1.5×10^6 cpm from kidney; autoradiography took 12 hr for liver samples and 36 hr for kidney samples. (*B*) Input of 5×10^6 cpm of liver nuclear RNA hybridized to three regions of mC/EBP cDNA (*insert*). (*C*) Input of nuclei from brain and kidney of 5×10^6 cpm and from fat and spleen of 1.5×10^6 cpm were hybridized; autoradiographic exposure was performed for 16–36 hr. Nuclei were prepared as described (20, 21) by using 0.5% Nonidet P-40 for spleen and 0.25% Triton X-100 for fat nuclei in the lysis solution.

evidence for hybridization only to one specific band, indicating that a single gene encodes this protein in mice as already described for rats (14). Finally, a transcription experiment in which the cDNA was divided into three parts was carried out (Fig. 2*B*). Each of the three segments of the cDNA gave an approximately equimolar signal, ruling out the presence of a localized repetitive sequence as the basis for the signal observed. Also this experiment detected no excessive 5′-end transcription that is characteristic of premature termination (24, 25). To investigate further the cell specificity of transcription of the mC/EBP gene, additional experiments with nuclei from brain, fat, and spleen cells as well as kidney and liver were carried out (Fig. 2 *B* and *C*). The transcriptional signal for mC/EBP was consistently strongest in the liver nuclei. Definite transcriptional signals were found also in fat and spleen nuclei but only low signals, just above background, in brain and kidney (Fig. 2 *B* and *C* and Table 1). By using either the input of total cpm or comparison with the signal for $tRNA^{Met}$, it appears that mC/EBP is transcribed about 30–40% as well in fat nuclei and 15% as well in spleen nuclei as it is in the liver. The transcription in total brain and kidney nuclei is no more than a few percent of that of liver.

In contrast to the large difference in mC/EBP transcription in different tissues, the gene for the widely distributed transcription factor Jun-B (23, 26) was transcribed at approximately similar rates in each of the tissues examined (Fig. 2 *B* and *C*; Table 1).

The differential transcription shown in these experiments is sufficient to explain the presence of the mC/EBP mRNA in hepatocytes and its near absence in Northern blots of total brain, spleen, or kidney mRNA. This in turn correlates with our inability to extract from these tissues a protein that binds to oligonucleotides recognized by mC/EBP (4). The presence of about 50% as much mRNA in fat cells as in liver cells was also reflected in the transcription rate in fat cells compared to hepatocytes. The very low but possibly real transcriptional signals in total brain tissue are in accord with the *in situ* hybridization results, which show the presence of mC/EBP mRNA in some brain cells.

Transcription Analysis in Cultured Primary Hepatocytes. When disaggregated hepatocytes are removed from animals and plated in culture as individual cells, the transcription rate of genes expressed in a liver-specific manner declines at least by a factor of 10 within the first 24 hr of culturing (20, 21). We have determined the rate of mC/EBP transcription under these circumstances (Fig. 3 *A* and *B*). Liver cells were dispersed by perfusion with EDTA and collagenase and were plated in culture for various periods. Nuclei were then collected to assay transcription from dispersed cells before and after culturing for various time periods. Albumin transcription was about 50% of the maximum level after 4 hr in culture and had decreased to about 10% after 24 hr in culture. The transcription rate of mC/EBP followed a pattern very similar to that of albumin. As we have pointed out in earlier work, this effect of cell dispersal on transcription by hepatocyte nuclei is not a general disruption of RNA polymerase II transcription but is specific to the differentiated hepatocyte profile (20, 21). For example, the transcription rate of some other genes remains unaffected or, in the case of actin,

Table 1. Relative transcription rates of mC/EBP and Jun B in various cell nuclei

	Liver				
	A	B	Brain	Spleen	Kidney
mC/EBP	46	43	1	7	2
Jun-B	13	86	9	11	16

Transcription assays were performed as described in Figs. 2 and 3. Numbers represent arbitrary units as defined in Fig. 2. Liver experiment A: the organs were removed and homogenized without perfusion as was the case for all other tissues. Liver experiment B: cells were isolated by perfusion of livers with collagenase before nuclei were prepared.

temporarily rises and then declines 24 hr after cells are put in culture (20, 21). There is still a strong actin signal in cells dispersed and cultured for 24 hr (Fig. 3). [In earlier experiments this signal had been shown to be about 3 times that of fresh liver nuclei (20, 21).] In addition to actin, we examined the transcription of the gene for the widely distributed Jun-B transcription factor in cultured hepatocytes. We knew that the transcription of this gene is stimulated in undifferentiated cells by serum and that its transcription rate goes up during liver regeneration (23, 26). There was a 5-fold increase in *Jun-B* transcription simply by perfusing the liver to remove the cells (Fig. 2*B*, Fig. 3, and Table 1). The transcription of *Jun-B* remained high for 24 hr (compare Fig. 2*B* with Fig. 3; see Table 1), even as the transcription rate for mC/EBP under the same conditions was falling. These results are strong evidence that the cell contact in the adult hepatocyte serves to provide signals that keep liver-specific gene transcription, including that of mC/EBP, at a high level. Just as important, cell–cell contacts probably prevent receptors that increase *Jun-B* transcription from being occupied and stimulating that gene.

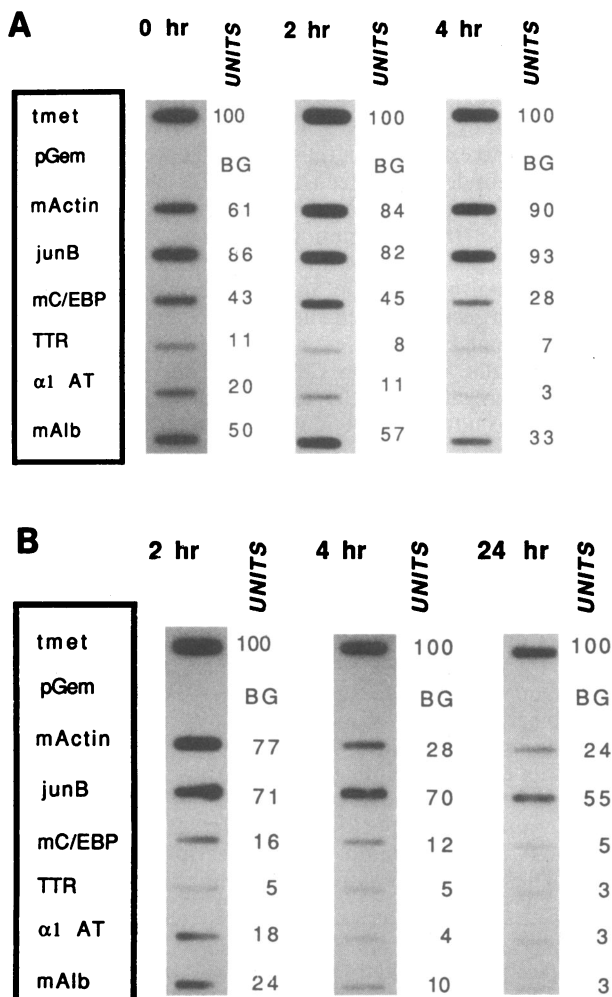

FIG. 3. Transcription of mC/EBP and other genes in cultured hepatocytes. Disaggregated hepatocytes (0 hr) and cells plated for the indicated times were used to assay transcription (21). The cloned DNA samples used were identified in Fig. 2. About 1.5×10^6 cpm of labeled nascent RNA was added in each experiment. Exposure time was between 24 and 48 hr.

DISCUSSION

The results we have presented bear on mammalian cell-specific transcriptional control in several ways. This is the first demonstration of a cell-specific transcriptional regulation of a mammalian nuclear DNA-binding factor that participates in coordinated cell-specific regulation. Other DNA-binding factors have been found that are limited in cell distribution (27–30) and may also be controlled at the level of transcription, but this has not yet been demonstrated. Cascades of regulated transcription factors have proved to be important in early larval embryogenesis in *Drosophila* (31), and this is likely to be true for transcriptional regulators associated with mammalian differentiation. In studying cell-specific factors that regulate genes in terminally differentiated cells, we presumably start at the end of a cascade of developmentally controlled genes. There must be other earlier acting transcription factors that regulate mC/EBP in a cell-specific manner, and these can now be sought with some assurance. The second conclusion about "cell specificity" that these results point to is that very few genes (and probably even fewer genes encoding transcription factors) are active exclusively in a single cell type. For example, TTR is transcribed in liver and choroid plexus (32) and α1-AT is transcribed in liver and macrophages (33). Apolipoprotein C is transcribed in both liver and intestine (34), and the complex of enzymes of fatty acid synthesis are prominent in liver, fat, and intestinal cells and also could be under transcriptional control (35). Thus, it is no surprise that mC/EBP is not confined only to hepatocytes. However, it is clearly not transcribed equally well in all cell types and therefore qualifies as a cell-limited if not a cell-specific factor that is itself under transcriptional control.

Finally, these experiments illustrate that some extracellular signals must exist for the active transcription of mRNAs encoding transcription factors even in end-stage differentiated cells. Ultimately, the connection must be made between these extracellular signals and the cascade of cell-specific or cell-limited factors that govern transcription. When additional "liver-specific" (or liver-enriched) transcription factors can be studied at the transcriptional level, we should begin to make progress on whether these extracellular signals are multiple as well as the factors that they regulate.

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