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Two Genetically Defined *Trans*-Acting Loci Coordinately Regulate Overlapping Sets of Liver-Specific Genes

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Summary

Mice homozygous for deletions around the albino locus fail to activate expression of a set of neonatal liver functions and die shortly after birth. This phenotype is thought to result from the loss of a positive *trans*-acting factor, denoted *alf*, in deletion homozygotes. Using differential cDNA screening, we isolated and characterized genes whose cell type-specific transcription is affected by *alf* and found as a common feature that expression of these genes is induced by glucocorticoids and cAMP. Surprisingly, a subset of these *alf*-responsive genes is negatively controlled by the tissue-specific extinguisher locus *Tse-1*. Administration of glucocorticoids and cAMP leads to reversal of *Tse*-1-mediated extinction of these genes. These results show that two *trans*-acting factors coordinately regulate expression of overlapping sets of liver-specific genes. We suggest that both the lethal phenotype and the extinguished state result from interference with hormone signal transduction.

Introduction

Multicellular organisms develop from a single cell, the zygote, which gives rise to a variety of cell types with different structures and functions. To understand the differentiation processes that yield these various phenotypes it is necessary to elucidate mechanisms by which genes are selectively expressed. Of particular importance for the establishment of a given pattern of gene activity is the interplay between controlling genes and signaling molecules such as hormones. Furthermore, it seems clear that tissue-specific patterns of gene activity depend on both positive and negative regulatory factors.

The tyrosine aminotransferase (TAT) gene is an example of a gene whose expression is controlled by positive and negative factors and by hormones. TAT expression is regulated by two genetically defined, *trans*-acting loci (Gluecksohn-Waelsch, 1979; Killary and Fournier, 1984) and is induced by both glucocorticoids and glucagon acting via cAMP (Granner and Beale, 1985). The TAT enzyme participates in hepatic gluconeogenesis and is synthesized exclusively in parenchymal cells of liver. Although enzyme synthesis normally rises within the first few hours after birth (Greengard, 1970), synthesis can be induced prematurely by administration of glucagon, which increases cAMP levels. This signaling molecule may play a role in the developmental activation of this gene, suggesting that the gene may be primed for expression in hepatocytes before hormones induce developmental activation (Greengard, 1969; Yeoh et al., 1979).

Albino mice homozygous for specific chromosome 7 deletions fail to activate a group of liver enzymes at birth, with resulting perinatal lethality. This suggests that a positive *trans*-acting factor required for expression of these genes is encoded in the deleted region (Gluecksohn-Waelsch, 1979). TAT and phosphoenolpyruvate carboxykinase (PEPCK) activities, two of the affected enzymes, are reduced along with the corresponding mRNAs (Schmid et al., 1985; Loose et al., 1986). Inducibility of the TAT gene by glucocorticoids and cAMP is absent in deletion homozygotes (Schmid et al., 1985). The effects of the albino lethal deletions are highly specific, and most liver functions assayed are not affected (Gluecksohn-Waelsch, 1979). Furthermore, 2D-gel analysis of proteins from normal and mutant mice indicate that levels of relatively few liver proteins were affected (Baier et al., 1984). Ultrastructural abnormalities were observed in the liver and proximal tubules of the kidney, suggesting that the mutation also influenced gene expression in kidney (Trigg and Gluecksohn-Waelsch, 1973). At least one of the affected enzymes, PEPCK, is known to be expressed in liver and in the proximal tubules of kidney (Meisner et al., 1985). Thus, the regulatory factor, which we denote *alf* (factor indicated by the albino lethal mutation), appears to be important for the biochemical maturation of hepatocytes and cells of the proximal tubules of the kidney.

A second locus that affects TAT gene activity has been identified by analyzing somatic cell hybrids between hepatoma cells and fibroblasts (Schneider and Weiss, 1971; Killary and Fournier, 1984). In hepatoma hybrids retaining mouse chromosome 11 or a specific region of human chromosome 17, TAT and PEPCK gene expression is selectively repressed, while expression of other liver genes is unaffected (Lem et al., 1988). This *trans*-dominant effect is mediated by a genetic locus designated tissue-specific extinguisher 1 (*Tse-1*) (Killary and Fournier, 1984). Another extinguisher locus (*Tse-2*) that maps to a different mouse chromosome (Petit et al., 1986; Chin and Fournier, 1989) selectively affects albumin and alcohol dehydrogenase.

It is not known how *alf* acts to control cell- and developmental-specific expression of the TAT gene and what role glucocorticoids and cAMP play in this induction. As indicated by the fact that TAT expression is not inducible in mutant mice, we hypothesized that the inducers cooperate with the *trans*-acting factor *alf* (Schmid et al., 1985; Gluecksohn-Waelsch, 1987). This hypothesis is supported by the finding that glucocorticoid induction, but not metal induction of the metallothionein gene, is affected in livers from mutant mice (DeFranco et al., 1988).

Is the coordinate regulation of TAT and PEPCK genes

by *alf* and *Tse-1* coincidental, or does this imply that both loci function in establishing liver-specific expression of these genes? To approach this question we used differential cDNA hybridization procedures to isolate cDNA clones whose expression was strongly affected by *alf*. Surprisingly, we found that many of the affected genes were also regulated by *Tse-1*, suggesting that the two *trans*-acting loci coordinately regulate expression of overlapping sets of liver-specific genes.

Results

Isolation and Identification of cDNA Clones Representing Genes Affected by the Albino Lethal Mutation

It seemed reasonable to assume that the albino lethal mutation might affect genes in addition to those identified previously by enzyme assays (Gluecksohn-Waelsch, 1979). To identify such genes by an unbiased approach, differential cDNA screening was performed. A cDNA library representing liver mRNA from normal mice was constructed and screened differentially with probes representing the mRNA populations from normal and mutant mice. Cross-hybridization experiments allowed grouping of 21 identified clones into nine distinct sequence families, designated X1 to X9, which by Southern blot analyses hybridized to different genomic DNA fragments and thus represent independent genes (data not shown). None of the transcripts encoded by X1 to X9 mapped to mouse chromosome 7 in the region of the albino lethal deletions; thus, none encodes the putative *trans*-activator itself (data not shown).

The DNA sequence of one member of each family was partially determined and compared with sequences in GenBank. No obvious sequence homology was evident for the cDNA clones X1, X2, X5, X7, or X9. Clone X3 was homologous to the rat PEPCK gene (Beale et al., 1985) and represented a full-length cDNA copy of this gene. PEPCK was already known to be influenced by the albino lethal deletion (Loose et al., 1986). X4 was more than 90% homologous to the rat α-fibrinogen gene (Crabtree et al., 1985). The cDNA insert of X6 showed >85% sequence identity with two rat serine protease inhibitors (Yoon et al., 1987; Le Cam et al., 1987). The cDNA clone X8 is a full-length cDNA copy of the mouse homolog of the rat aldolase B gene (ALDB) (90% sequence identity) (Tsutsumi et al., 1984). The effects of albino lethal deletions on ALDB expression had been reported previously (Sala-Trepat et al., 1985).

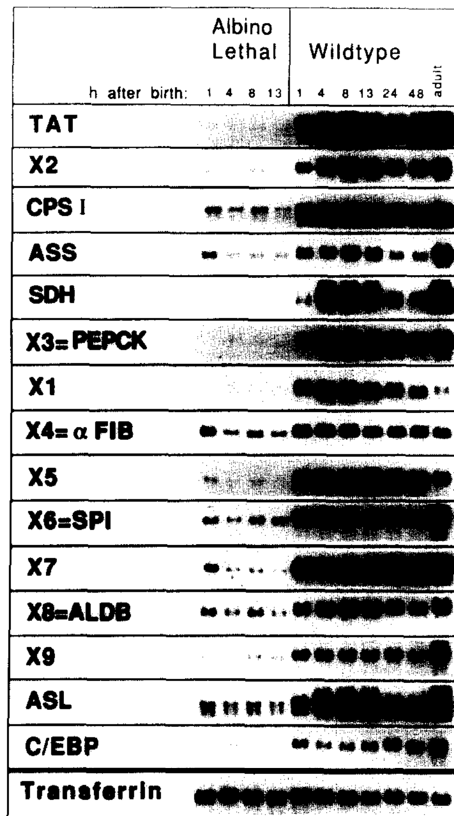

Figure 1. Postnatal Regulation of Gene Expression in Albino Lethal Mice and Normal Littermates

Total RNA (5 μg), isolated from the livers of albino lethal mice and from normal littermates at the indicated time points after birth, were analyzed by Northern blot hybridization with various cDNA probes (see Experimental Procedures). The following abbreviations are used: TAT (tyrosine aminotransferase), CPSI (carbamoylphosphate synthetase I), ASS (argininosuccinate synthetase), SDH (serine dehydratase), PEPCK (phosphoenolpyruvate carboxykinase), αFIB (α chain of fibrinogen), SPI (serine protease inhibitor), ALDB (aldolase B), ASL (argininosuccinate lyase), C/EBP (CCAAT box/enhancer binding protein). X1 to X9 designate the mouse cDNA clones isolated by subtractive cDNA hybridization. The autoradiograms were exposed for different amounts of time. All filters were rehybridized with either a glyceraldehyde-3-phosphate-dehydrogenase cDNA probe (Fort et al., 1985) or with a transferrin cDNA probe as an internal standard (data not shown).

Expression of a Group of Liver Genes Is Affected by the Albino Lethal Mutation

To confirm that expression of the mRNAs represented by cDNA clones X1 through X9 was affected by the albino lethal deletion, we analyzed expression patterns in the livers of newborn mice. We also included probes for urea cycle enzymes (carbamoylphosphate synthetase I [CPSI], argininosuccinate synthetase [ASS], and argininosuccinate lyase [ASL]) in this analysis because these enzymes are affected in mutant livers (Morris et al., 1988) and, like TAT (see Introduction), depend on glucocorticoids and cAMP for maximal synthesis (Nebes and Morris, 1988). RNAs were isolated from the livers of deletion homozygotes and from normal littermates at various times after birth. As shown in Figure 1 synthesis of all these mRNAs was reduced in the albino liver. Even 13 hr after birth the level of the mRNAs encoding these proteins did not increase in albino mutants, arguing against mere delayed activation of these genes. Low levels of the mRNAs encoding TAT, PEPCK, ALDB, SDH (serine dehydratase), and three urea cycle enzymes were predicted from previous work (Schmid et al., 1985; Loose et al., 1986; Sala-Trepat et al., 1985; Gluecksohn-Waelsch et al., 1974; Morris et al., 1988). Interestingly, expression of the gene encoding a DNA binding protein isolated from liver, C/EBP (CCAAT box/enhancer binding protein) (Land-

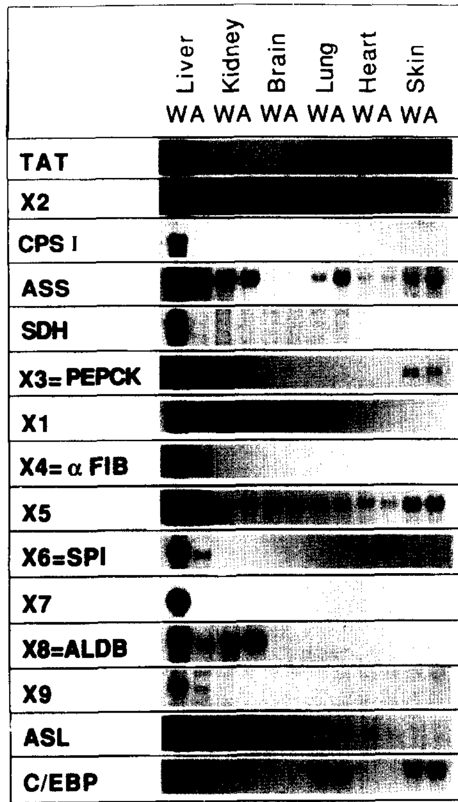

Figure 2. Tissue-Specific Expression of the Albino Lethal Phenotype
Total RNA (5 μg) isolated at 2–5 hr after birth from the indicated tissues/organs of albino lethal mice (A) and from their normal littermates (W, wild-type) were analyzed by Northern blot hybridization with various cDNA probes (see Experimental Procedures and Figure 1 for probes and abbreviations). The autoradiograms were exposed for different times. All filters were rehybridized as described in Figure 1.

schulz et al., 1988), was also influenced by the albino lethal deletion (Figure 1 and below).

The Effect of the *Trans*-Activator *alf* Is Cell Type–Specific

The albino lethal deletions affect the livers and kidneys of mutant mice, but other organs do not display morphological or ultrastructural abnormalities (Gluecksohn-Waelsch, 1979). We consequently determined whether expression of the RNAs affected in liver was affected in other organs. RNAs were isolated from a variety of tissues of albino lethal and normal mice and hybridized with labeled cDNA probes. As shown in Figure 2, most of the affected genes were expressed preferentially or exclusively in liver. Expression in other tissues was not affected by the deletion, with two exceptions. Expression of the genes encoding X1 and X3 (PEPCK) was affected in both liver and kidney. However, PEPCK gene expression was not affected in skin. Expression of ALDB, which occurs in liver and kidney, was affected only in liver. The most remarkable expression patterns were observed for the X5, ASS, and ASL genes. These mRNAs are expressed in all tissues analyzed. However, the effect of the albino lethal deletion was only apparent in the liver; expression in kidney, brain, heart, lung, and skin of normal and mutant mice was comparable. Also, expression of C/EBP in lung and skin was *alf*-independent; reduced expression was only evident in the mutant liver.

To define the cell types in which *alf*-responsive genes were expressed, we analyzed TAT, PEPCK, ALDB, and X1 expression in livers of normal and mutant mice by in situ hybridization. Expression was restricted to parenchymal cells (Figure 3 and data not shown) and was dramatically reduced in mutant livers. The liver-specific transferrin gene was expressed to the same extent in albino lethal and normal mice. The surprising finding that a subset of genes (X1 and PEPCK) affected in the liver was also affected in the kidney prompted us to analyze the cells in which they are expressed. Expression of X1 and PEPCK required *alf* in the kidney and was restricted to the proximal tubular cells, whereas expression of ALDB not affected by *alf* also occurred in other kidney cells (data not shown). Interestingly, PEPCK expression in the kidney can be induced with glucocorticoids (Meisner et al., 1985), whereas ALDB gene expression in kidney is constitutive (Munnich et al., 1985).

Expression of *alf*-Dependent Genes Is Inducible by Hormones and Controlled at the Transcriptional Level

We have previously observed (Schmid et al., 1985) that responsiveness of the TAT gene in albino lethal mice to glucocorticoids and cAMP is impaired. Therefore, we asked whether the entire set of genes affected by *alf* is responsive to these inducers. The results summarized in Figure 4 demonstrate that all *alf*-responsive genes except C/EBP responded to these inducers. This finding further supports the hypothesis of an interdependence of *alf* and the proteins involved in mediating the effects of these inducers on transcription (Schmid et al., 1985; Gluecksohn-Waelsch, 1987).

Nuclear run-on transcription assays using nuclei from livers of mutant and normal mice indicated that the relative transcription rates of *alf*-responsive genes were reduced in mutant livers (data not shown). This demonstrates that a strongly reduced transcriptional activity is responsible for the low mRNA levels in the livers of albino lethal mice.

The Albino Lethal Deletion Affects the Expression of the DNA Binding Protein C/EBP

The finding that expression of a set of liver-specific genes was affected in albino lethal mice suggested that these genes might share a common regulatory factor(s). Furthermore, since most of these genes are inducible with glucocorticoids and cAMP (Figure 4), responses that are deficient in albino lethal mice (Schmid et al., 1985), we considered the possibility that components of the induction pathway(s) might be deficient in deletion homozygotes. Therefore, we analyzed expression of regulatory proteins mediating the hormonal effects: the glucocorticoid receptor, the cAMP-dependent protein kinase A, and the cAMP response element binding protein (CREB) (Figure 5). Expression of mRNAs encoding these regulatory factors was not affected in livers of deletion homozygotes.

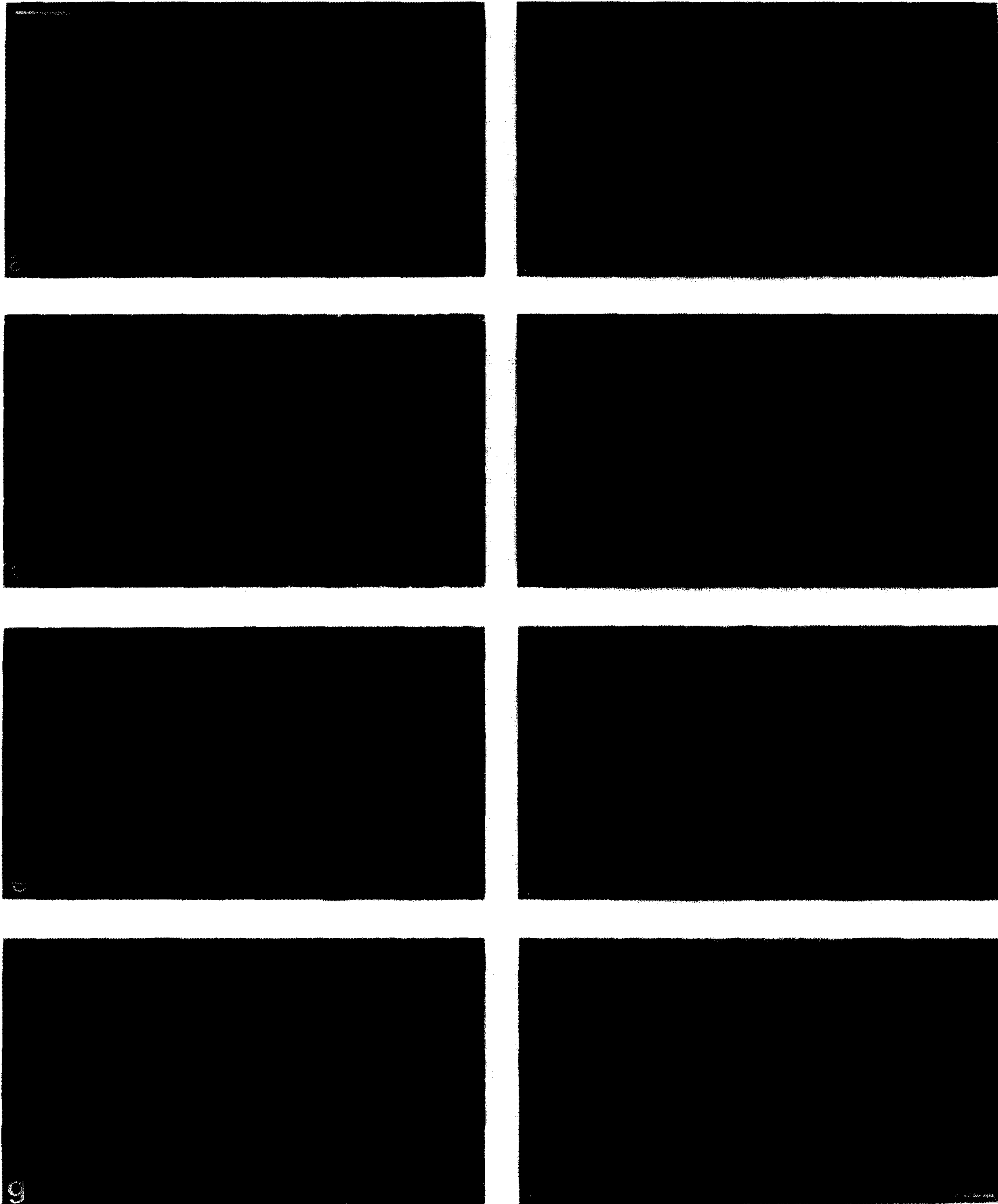

Figure 3. The Albino Lethal Deletion Affects Gene Expression in a Cell Type-Specific Manner

Analysis of TAT (a–f) and transferrin (g and h) gene expression in wild-type and mutant livers by in situ hybridization. Parenchymal cells of the livers (weakly stained with hematoxylin and eosin) show hybridization with the ^{32}P-labeled cRNA probe. Nonhepatocyte cells (darkly stained) do not express these genes. Identity of the cells has been confirmed by immunohistochemical analyses (W. W. Franke and C. Kuhn, personal communication). (a and b), bright-field illumination, bar corresponds to 30 μm; (c and d), survey autoradiograph of (a) and (b), (dark-field illumination); (e–h), higher magnifications, bar corresponds to 12 μm.

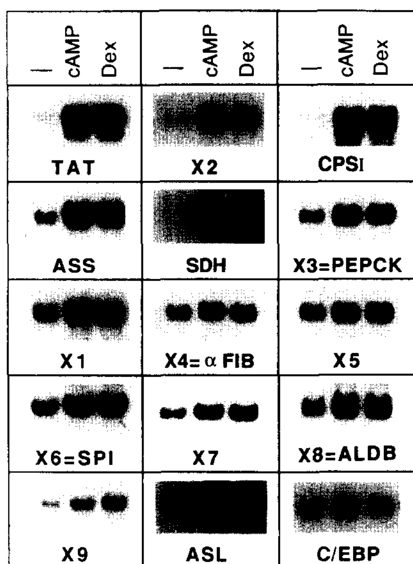

Figure 4. The Genes Responsive to *alf* Are Inducible by cAMP and Glucocorticoids

Five micrograms of pooled RNAs from 1-day-old newborn wild-type mice injected with either cAMP (cAMP) for 2 hr or with dexamethasone (Dex) for 4 hr or untreated (−) were analyzed by Northern blot hybridization with the indicated cDNA probes (see Figure 1 and Experimental Procedures for abbreviations and probes). The autoradiograms were exposed for different times. Filters were rehybridized as described in Figure 1.

As affected genes are expressed predominantly in liver, we analyzed expression of the genes encoding the transcription factors C/EBP and HNF-1, which were recently purified from liver (Landschulz et al., 1988; Frain et al., 1989). Analysis with cloned DNA of the transcription factor HNF-1 (also referred to as LF-B1 [Frain et al., 1989]), involved in expression of several liver-specific genes (Courtois et al., 1988), showed no difference in liver mRNA levels in wild-type and mutant mice (data not shown). However, expression of C/EBP mRNA was reduced in livers of albino lethal mice (Figures 1 and 5). Interestingly, expression of C/EBP was affected by *alf* in the liver but not

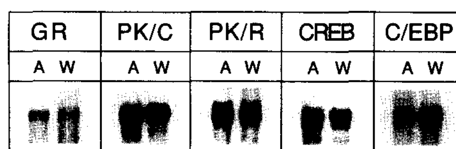

Figure 5. Analysis of Expression of Regulatory Proteins in Normal and Mutant Mice

Five micrograms of poly(A)$^+$ RNA isolated from the livers of albino lethal mice (A) and from their normal littermates (W, wild-type) were analyzed by Northern blot hybridization with the indicated cDNA probes (for details, see Experimental Procedures). The following abbreviations were used: GR (glucocorticoid receptor), PK/C (catalytic subunit of the cAMP-dependent protein kinase A), PK/R (regulatory subunit of the cAMP-dependent protein kinase A), CREB (cAMP response element binding protein), C/EBP (CCAAT box/enhancer binding protein). The autoradiograms were exposed for different times. Filters were rehybridized as described in Figure 1.

Table 1. C/EBP Does Not *Trans*-Activate the TAT Gene

Plasmid	pHD	pHD-C/EBP	Foldness
pBLCAT5[a] (=TKCAT)	3.3	6.3	1.9
D9(Alb)[b]	13.4	306.3	22.9
TTC337[c]	180.8	353.8	1.9
TATCAT −3341/+62[c]	0.7	1.1	1.5
TATCAT −3922/+62[c]	21.0	26.2	1.2
5× BI[c]	49.6	43.6	0.9
5× BIII[c]	335.4	532.8	1.6

The indicated reporter plasmids were cotransfected into FTO2B cells together with either the control plasmid pHD (Müller et al., 1988) or the C/EBP expression plasmid pHD-C/EBP. After 65 hr, extracts were prepared and assayed for CAT activity. Absolute CAT expression is given as pmol/min per mg.

[a] pBLCAT5 is a modification of pBLCAT2 (Luckow and Schütz, 1987), where two SV40 polyadenylation signals replace vector sequences upstream of the multiple cloning site (M. B., unpublished data).

[b] Nine copies of the albumin promoter D element (Maire et al., 1989) cloned in front of the TK promoter in pBLCAT5.

[c] These constructs are described in Boshart et al. (1990) and were modified as indicated for pBLCAT5.

in other organs (Figure 2). Since albino lethal mice do express detectable C/EBP mRNA, this gene is not encoded at the vicinity of the albino locus.

As C/EBP might be involved in mediating the *alf* response, we tested, by performing cotransfection experiments (Table 1), whether C/EBP could activate transcription via TAT regulatory sequences. The expression vector pHD-C/EBP, encoding full-length C/EBP, conferred a strong and specific effect via the D element of the albumin promoter (Maire et al., 1989; Friedman et al., 1989), which we used as positive control (construct D9(Alb) in Table 1). However, C/EBP did not activate transcription from TATCAT constructs. TATCAT −3922/+62 (but not TATCAT −3341/+62) includes the hepatocyte-specific enhancer described in Boshart et al. (1990). Also, when we tested a 337 bp fragment spanning this enhancer (construct TTC337) and the two essential multimerized enhancer elements (constructs 5× BI and 5× BIII) in front of the TK promoter, no *trans*-activation by C/EBP was detectable (Table 1).

A Subset of *alf*-Responsive Genes Is Also Regulated by *Tse-1*

Somatic cell hybrids between hepatoma cells and fibroblasts fail to express liver functions—a phenomenon termed extinction. Extinction has been shown to have a specific genetic basis (Killary and Fournier, 1984). For example, it has been shown that the presence of a segment of human chromosome 17 is sufficient to extinguish TAT and PEPCK expression in rat hepatoma cells, suggesting that a single genetic locus, denoted *Tse-1* (tissue-specific extinguisher 1), is responsible for extinction of these genes (Lem et al., 1988). Since TAT and PEPCK both require *alf* for liver-specific expression, we hypothesized that other genes affected by *alf* might also be regulated by *Tse-1*. Therefore, we measured mRNA levels of these genes in hepatoma deletion hybrids retaining fragments of human fibroblast chromosome 17 including or not including the *Tse-1* locus

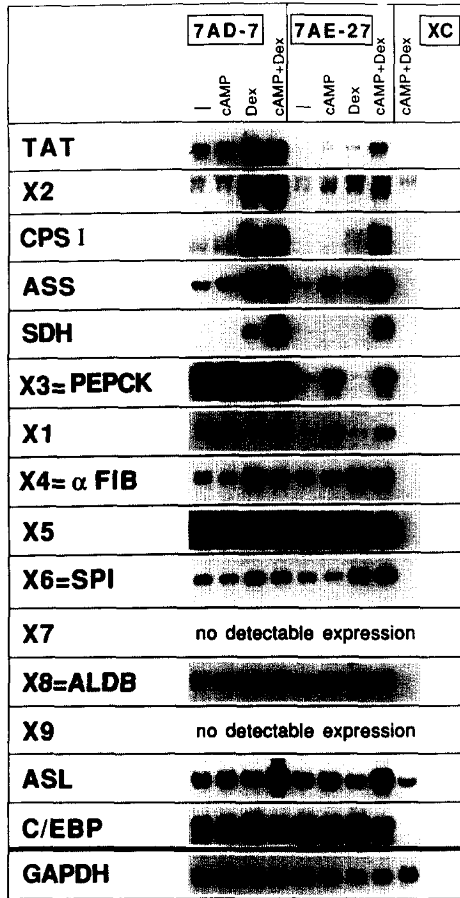

Figure 6. A Subset of Genes Affected by the Albino Lethal Mutation Is Also Controlled by *Tse-1*

The indicated cell lines were either mock-induced (–) or induced with cAMP (cAMP), with dexamethasone (Dex), or induced with both cAMP and dexamethasone (cAMP + Dex). The XC cell line, included as a control, was induced with cAMP and dexamethasone. Ten micrograms of total RNA derived from these cell lines was analyzed by Northern blot hybridization with the cDNA probes described in Figure 1. The filters were rehybridized with a GAPDH-encoding cDNA (Fort et al., 1985).

(Figure 6). Clone 7AE-27 retains several human chromosome 17 markers including human *Tse-1*. 7AD-7 retains a subset of these markers but is *Tse-1*$^-$ (Lem et al., 1988). Interestingly, several *alf*-responsive genes are responsive to the presence of *Tse-1* (Figure 6, compare lanes 1 and 5). Since X7 and X9 were not expressed in the *Tse-1*$^-$ cell line, their response to *Tse-1* could not be ascertained. Thus, a subset of *alf*-responsive genes is negatively regulated by *Tse-1* in hepatoma hybrids.

Previous observations (Thayer and Fournier, 1989) and the interesting finding that *Tse-1* acts through the cAMP response element of the TAT gene (see Discussion and Boshart et al., 1990) raised the question whether hormonal inducers could antagonize the effect of *Tse-1* on this subset of genes. Therefore, we determined mRNA levels in the two cell lines after induction with cAMP or dexamethasone or both inducers (Figure 6). The negative regulatory effect in the *Tse-1*$^+$ cell line (7AE-27) can be almost completely reversed by either cAMP alone (e.g., PEPCK) or by cAMP together with glucocorticoids (e.g., TAT). In a different *Tse-1*$^+$ hybrid clone, extinction of the TAT gene was reversed by cAMP induction alone (Thayer and Fournier, 1989). Thus, a functional antagonism between negative regulation by *Tse-1* and hormone induction appears to be characteristic of genes affected by the *Tse-1* locus. This antagonism may play a role in the hormone-triggered perinatal activation of several liver genes.

Discussion

The Albino Lethal Mutation Affects Expression of Genes in Liver and Kidney

The phenotype of mice homozygous for deletions around the albino locus on chromosome 7 provides evidence for the existence of a *trans*-acting factor (*alf*, factor indicated by the albino lethal mutation) involved in the control of a number of liver genes. We used differential cDNA hybridization techniques to isolate a representative sample of affected genes in addition to those that have been previously identified by enzymatic assays (Gluecksohn-Waelsch, 1979). All of the cDNAs we identified were expressed preferentially or exclusively in liver. Furthermore, expression was affected by the albino lethal deletion in liver and in two cases in kidney, but not in other organs. This suggests that *alf* is required in liver and kidney. By in situ hybridization, tissue-specific regulation has been further limited to parenchymal cells of the liver and to proximal tubular cells of the kidney (Figure 3 and data not shown). Ultrastructural abnormalities are restricted to these cells in the mutant mouse (Trigg and Gluecksohn-Waelsch, 1973). Furthermore, the genes affected by *alf* respond to glucocorticoids or cAMP (Figure 4; Granner and Beale, 1985; Munnich et al., 1985; Nebes and Morris, 1988; Noda et al., 1988), whereas genes (e.g., albumin) not affected by the albino lethal deletion do not exhibit any inducibility by glucocorticoids or via cAMP (data not shown).

As cAMP and glucocorticoid inducibility of TAT in liver is lost in newborn deletion homozygotes, it has been suggested (Schmid et al., 1985; Gluecksohn-Waelsch, 1987) that *alf* might interact with components of the induction pathways. Our data show that mRNAs encoding the glucocorticoid receptor, protein kinase A regulatory and catalytic subunits, and CREB are not affected by *alf*. Recently, it has been shown that the glucocorticoid receptor cooperates in a cell type–specific fashion with different transcription factors for maximal activity (Strähle et al., 1988), suggesting that *alf* might play a similar role.

Interestingly, the level of C/EBP mRNA coding for a transcription factor (Landschulz et al., 1988; Friedman et al., 1989) is reduced in livers of newborn deletion homozygotes. It was thus conceivable that C/EBP is involved in a cascade of events by which *alf* regulates its target genes. We therefore analyzed whether C/EBP would *trans*-activate the TAT gene. No effect on TAT regulatory sequences was observed under conditions leading to a potent *trans*-activation of a cognate C/EBP binding site from the albumin promoter (D element; Maire et al., 1989). Thus, we wish to conclude that C/EBP does not play a direct role in determining liver-specific expression of TAT.

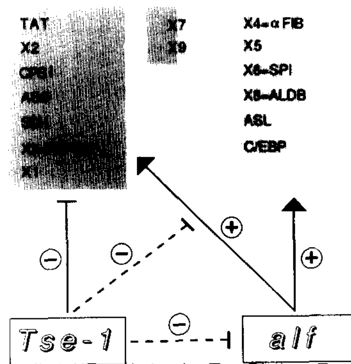

Figure 7. Coordinate Regulation by Two Distinct *Trans*-Acting Loci

One set of genes (dark-shaded) is regulated by both *Tse-1* (encoded on mouse chromosome 11 or human chromosome 17) and *alf* (encoded on mouse chromosome 7). Expression of the second set of genes (light-shaded) is not affected by *Tse-1*. The genes coding for X7 and X9 are affected by *alf*. Since these genes were not expressed in any cell line analyzed (Figure 6 and data not shown), evidence for coregulation by *Tse-1* cannot be established. Solid and dashed lines indicate possible modes of interaction between *Tse-1* and *alf* (see Discussion). Abbreviations are described in Figure 1.

Overlapping Sets of Genes Are Regulated by Two *Trans*-Acting Loci

A subset of genes positively regulated by *alf* in liver was also negatively regulated in hepatoma hybrids by the tissue-specific extinguisher 1 (*Tse-1*). Thus, two loci involved in the control of liver-specific gene expression affect distinct but overlapping sets of liver genes (Figure 7). This finding provides evidence for coordinate control of sets of liver genes by common regulators and suggests that a specific pattern of properly timed, cell lineage–specific expression might require control by more than one *trans*-acting factor. Thus, the specific regulatory pattern of a given gene seems to be the consequence of coregulation by specific combinations of *trans*-acting factors (Serfling et al., 1985).

In line with this reasoning, liver-specific expression of the α-fibrinogen gene seems to be dependent on an intact binding site for the liver-specific transcription factor HNF-1 (Courtois et al., 1987) and is also regulated by *alf*, as shown in this report. HNF-1 is involved in liver cell–specific transcriptional control of a group of liver genes, most of which are not regulated by *alf* nor by *Tse-1* (Courtois et al., 1988; Frain et al., 1989). The concept that a characteristic regulatory pattern is generated by a specific combination of *trans*-acting loci parallels the well-established modular structure of *cis*-acting transcriptional control elements, where specific regulatory properties are thought to be determined by a characteristic combination of a limited number of transcription factor binding sites (modules). A given module is usually part of the regulatory elements of several or even many genes.

Hormone Induction Leads to Reversal of Extinction

We have shown that reversal of *Tse-1*–mediated extinction by hormones (see also Thayer and Fournier, 1989) can be observed for all *Tse-1*–responsive genes identified so far. Thus, a more direct link between *Tse-1* function and signal transduction pathways mediating hormone action seems likely. As shown in the accompanying paper (Boshart et al., 1990) *Tse-1* acts by interference with the cAMP signal transduction pathway. Thus, reversal of extinction by hormone induction seems to reflect a direct antagonism between *Tse-1*–mediated repression and cAMP action. It is tempting to speculate that this functional antagonism is relevant for the activation of the TAT gene in vivo.

One attractive model would give *Tse-1* a central role in prenatal repression of a set of genes that is *alf*-dependent and known to be switched on perinatally by the strong release of gluconeogenic hormones resulting from neonatal hypoglycemia. Activity of *Tse-1* in the liver would gradually decrease during liver development, rendering the set of repressed genes increasingly responsive to hormonal stimulation toward the end of gestation. In fact, TAT as well as SDH can be induced prematurely—days before birth—by administration in utero of glucagon (acting via cAMP) (Greengard, 1970), and this effect is enhanced by glucocorticoids (Ruiz-Bravo and Ernest, 1982).

Tse-1 and *alf* Regulate Their Target Genes Independent of Each Other

For most of the genes affected by the albino lethal deletion, transcription rates have been measured in normal and mutant mice (data not shown and Morris et al., 1988). Using homologous cDNA clones it could be shown that *alf*-mediated regulation of TAT, PEPCK, and all the genes analyzed in this study is clearly at the level of transcription. Transcriptional control has also been documented by run-on experiments for the dominant negative regulation by *Tse-1* and reversal of extinction in the hepatoma hybrid line used in this work (E. Schmid and A. F. Stewart, unpublished data). We do not know, however, whether *alf* and *Tse-1* exert their regulatory effects directly, e.g., as transcription factors. One or several intermediate steps might be involved. Extinction of growth hormone expression in somatic cell hybrids has recently been shown to be a consequence of down-regulation of the cell-specific transcription factor GHF-1, which is required for growth hormone expression (McCormick et al., 1988).

Thus, the dominant negative control operates through repression of a positive regulator. *alf* is unlikely to be the target of dominant negative regulation by *Tse-1*, as only a subset of *alf*-regulated genes is extinguished by *Tse-1*. To understand how the two *trans*-acting loci contribute to the realization of a cell-specific and properly timed gene expression program, we are now focusing our efforts on cloning both *alf* and *Tse-1*.

Experimental Procedures

Outline of Subtractive cDNA Hybridization

To identify a representative collection of genes that are affected by the albino lethal mutation, differential cDNA screening was performed. A cDNA library representing liver mRNA of c^{ch}/c^{ch} normal mice was constructed. Duplicate filters of this library were hybridized with either mRNA probes or cDNA probes, representing the mRNA population from normal (c^{ch}/c^{ch}) and mutant (c^{3H}/c^{3H}) mice. To intensify differential signals, both probes and the cDNA library filters were prehybridized with cDNA fragments isolated from a mutant (c^{3H}/c^{3H}) liver cDNA library. These steps are outlined in the sections below. Candi-

date clones giving a stronger signal with probes derived from normal mice were plaque purified.

Construction of cDNA Libraries

Poly(A)$^+$ RNA was prepared from livers of newborn albino lethal (c^{3H}/c^{3H}) mice and from their normal littermates (c^{ch}/c^{ch} or c^{ch}/c^{3H}). cDNA libraries in λgt10 (Huynh et al., 1985) were constructed following the protocol by Gubler and Hoffman (1983) with modifications (Ruppert et al., 1988). The newborn c^{ch}/c^{ch} mouse liver cDNA library (average insert size 1.6 kb) contained 2×10^7 recombinants of which 5×10^5 pfu were amplified on Escherichia coli C600hfl. Two newborn c^{3H}/c^{3H} mouse liver cDNA libraries each contained 5×10^7 recombinants of which 5×10^6 pfu (Pool I, 1.4 kb average insert size) and 3.8×10^6 pfu (Pool II, 0.85 kb average insert size) were amplified.

Preparation of Competitor DNA and Prehybridization of the cDNA Library

The EcoRI inserts of the c^{3H}/c^{3H} mouse liver cDNA library were cloned into the plasmid vector pSP64 (Melton et al., 1984) to generate a plasmid cDNA library of 1.5×10^6 recombinant colonies. The same cDNA fragments were also cloned into the phage plasmid vector M13mp8 (Messing and Vieira, 1982) to generate a library of 5×10^5 recombinants.

The c^{ch}/c^{ch} cDNA library was plated at a density of 1×10^5 phages per NUNC dish (22 cm × 22 cm), subsequently transferred in duplicates onto Gene Screen membranes (NEN), and covalently bound by UV cross-linking (Church and Gilbert, 1984). The duplicate filters were presaturated by an overnight incubation in 0.375 M Na_2HPO_4, 5.4% SDS (Bio-Rad), 15.4% deionized formamide (Fluka), 0.77% bovine serum albumin (BSA) (Sigma), and 1 mM EDTA containing either 320 µg/ml EcoRI-digested and denatured c^{3H}/c^{3H} competitor DNA or 600 µg/ml single-stranded c^{3H}/c^{3H} recombinant M13mp8 DNA.

Synthesis, Presaturation, and Hybridization of mRNA Probes

Labeling of poly(A)$^+$ RNA was started by resuspending 1.5 µg of poly(A)$^+$ RNA in 5 µl of 50 mM Tris-HCl (pH 9.5) followed by a 5 min incubation at 95°C. After addition of 5 µl of [γ-^{32}P]ATP in 50 mM Tris-HCl (pH 9.5), 20 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 0.5 µl of T4 polynucleotide kinase (PL-Biochemicals), the reaction was incubated for 30 min at 37°C and subsequently heat-inactivated. The labeled poly(A)$^+$ RNA was precipitated, washed in 80% ethanol, and resuspended in 130 µl of H_2O. Prehybridization of the labeled poly(A)$^+$ RNA from either c^{ch}/c^{ch} or c^{3H}/c^{3H} liver was performed in 0.375 M Na_2HPO_4, 5.4% SDS, 15.4% formamide, 0.77% BSA, and 1 mM EDTA containing 1 mg/ml denatured c^{3H}/c^{3H} competitor cDNA fragments at 61°C for 15 hr. The presaturated poly(A)$^+$ RNA was added directly to the hybridization mix. Hybridization was carried out in polypropylene cylinders rotating in an incubator (Bachofer GmbH, Reutlingen, Federal Republic of Germany) at 65°C for 36 hr. After hybridization the filters were washed in 25 mM Na_2HPO_4, 1% SDS, 1 mM EDTA (pH 7.2) at 65°C six times for 30 min each and exposed to Kodak X-OMATAR films.

Synthesis, Presaturation, and Hybridization of cDNA Probes

Synthesis of labeled cDNA was performed by incubating 2 µg of either c^{ch}/c^{ch} or c^{3H}/c^{3H} poly(A)$^+$ RNA in 30 µl of 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 10 mM DTT, 150 mM KCl, 500 µg/ml actinomycin D, 100 µg/ml oligo(dT), 1 mM dGTP, 1 mM dCTP, 20 µM dATP, 20 µM dTTP, 250 µCi of [α-^{32}P]dATP (410 Ci/mmol), 250 µCi of [α-^{32}P]dTTP (410 Ci/mmol), 0.5 U/µl RNasin, and 0.5 U/µl AMV reverse transcriptase for 2 hr at 42°C. The reaction was stopped by the addition of 10 mM EDTA and heat-inactivated. The poly(A)$^+$ RNAs were alkaline hydrolyzed. Labeled cDNAs (100 ng) were coprecipitated with 60 µg of single-stranded c^{3H}/c^{3H} recombinant M13mp8 DNA. After resuspension in 10 µl of H_2O, 10 µl of 2× hybridization mix (1 M Na_2HPO_4, 1 M NaH_2PO_4, 10 mM EDTA, 0.2% SDS) was added, and presaturation was performed for 65 hr at 68°C. The cDNA/M13mp8 hybrid molecules were separated from single-stranded cDNA by hydroxyapatite chromatography (Bio-Rad). The single-stranded fraction was resubtracted with c^{3H}/c^{3H} single-stranded M13mp8 recombinant DNA and proceeded as before. Labeled cDNAs were then hybridized to presaturated duplicate c^{ch}/c^{ch} filters as described above.

Analysis of the cDNA Inserts by Hybridization and DNA Sequencing

The inserts of 21 phages of the c^{ch}/c^{ch} λgt10 library, giving a stronger signal with probes derived from normal mice (c^{ch}), were isolated, subcloned into the plasmid vector Bluescript M13$^+$ (Stratagene), grouped into nine distinct cross-hybridizing families, and characterized by restriction enzyme analyses and DNA sequencing. Sequences obtained were compared with the DNA sequences of GenBank and are available on request.

Probes Used in the RNA Analyses

pmcTAT4 (S. R., unpublished data) contains a 1.0 kb EcoRI-BamHI fragment (position +20/+1020) of the full-length mouse TAT cDNA (S. R., unpublished data). prSDH (M. B., unpublished data) contains the large PstI fragment (780 bp) of pSDH4 (Noda et al., 1985). pAL1-2 and pAL0.5-2 (S. R., unpublished data) contain either the 1.0 kb or 0.5 kb EcoRI fragment of pAl-2, encoding rat ASL (provided by S. Manara Shediac). prASS-1 (M. B., unpublished data) contains a 1.5 kb PstI fragment, encoding rat ASS (provided by S. Morris). prCPSI-1 (M. B., unpublished data) contains a 4 kb EcoRI-BamHI fragment from the cDNA clone δCPSI encoding rat CPSI (provided by S. Morris). CαSP6 (provided by G. S. McKnight) contains a 500 bp 3′ fragment from MC-1 encoding in part the catalytic subunit of the mouse cAMP-dependent protein kinase. The clone RIαSP6 (provided by G. S. McKnight) encodes in part the regulatory subunit of the mouse cAMP-dependent protein kinase. pBS$^+$NC/EBP contains a full-length cDNA copy coding for C/EBP (Landschulz et al., 1988; provided by W. H. Landschulz and S. L. McKnight). The EcoRI-HindIII fragment of pBS$^+$NC/EBP was cloned in the expression plasmid pHD (Müller et al., 1988) to yield pHD-C/EBP. pmcGRH1 (S. R., unpublished data) contains the 1 kb HindIII fragment of the mouse glucocorticoid receptor cDNA clone (position +1319 to +2347) from pSV2Wrec (Danielsen et al., 1986). pmTF-1 (1.5 kb EcoRI fragment) and pmTF-2 (750 bp EcoRI fragment) are subclones of the full-length mouse transferrin cDNA (S. R., unpublished data), which have been identified by revealing more than 86% sequence homology to the human transferrin gene (Yang et al., 1984). pmcCREB61 (S. R., unpublished data) contains a 1.2 kb EcoRI fragment corresponding to the mouse cDNA encoding the CREB isolated by cross-hybridization with the human CREB cDNA clone (Hoeffler et al., 1988). pSP-GAPDH contains the 800 bp XbaI-PstI fragment of pRGAPDH-13 (Fort et al., 1985) cloned into the pSP64 vector (W. S., unpublished data). pm18S1,3XE (S. R., unpublished data), which has been used in the nuclear run-on experiment, contains the 1.3 kb XbaI-EcoRI fragment of λgtWES Mr974 (Grummt et al., 1979) corresponding to the 18S coding region. The plasmid clones X1 to X9 are described in this paper. All described fragments are cloned in either Bluescribe M13$^+$, Bluescript M13$^+$, pSP64, or pSP65 vectors and are used to generate in vitro radiolabeled antisense RNA transcripts by using either T3, T7, or SP6 RNA polymerases (Melton et al., 1984).

RNA Preparation and Northern Blot Analysis

Livers, kidneys, brains, hearts, lungs, and skin of 21 albino lethal mice (c^{14CoS}/c^{14CoS}) and of 21 normal mice (c^{ch}/c^{ch} and/or c^{ch}/c^{14CoS}) were pooled, and RNA was prepared according to Krieg et al. (1983). For the time course experiment the livers were taken at the indicated time points after birth (variations of 30 min are possible): 0–1 hr (10 albino lethal and 20 normal mice from 5 litters), 4 hr (15 albino lethal and 36 normal mice from 7 litters), 8 hr (9 albino lethal and 31 normal mice from 7 litters), 13 hr (11 albino lethal and 18 normal mice from 5 litters), 1 day (17 normal mice from 2 litters), 2 days (10 normal mice from 2 litters). To correct for different RNA yields per liver, each liver was used individually for RNA preparation, and 100 µg from each preparation was pooled for each time point. For the in vivo induction experiment five 1-day-old newborn mice were injected either for 4 hr with dexamethasone (100 ng/g body weight) or for 2 hr with chlorophenylthio-adenosine-3′:5′-monophosphate (final concentration 10^{-5} M) by intraperitoneal injection. Northern blot analysis was performed as described (Schmid et al., 1987).

In Situ Hybridization

Livers and kidneys were taken from 3 hr c^{14CoS}/c^{14CoS} and normal mice and were shock frozen in *iso*-pentane/liquid nitrogen. Hybridizations were performed on 5 µm cryostat sections as described by Leube et al. (1986) with the following modifications. The sections were fixed in 4% formaldehyde, acetylated, and prehybridized for 30 min at 50°C in 50% deionized formamide, 10% dextran sulfate, 4× SSPE, 10 mM Tris-HCl (pH 7.5), 0.1% SDS, 1× Denhardt's solution, 500 µg/ml E. coli

tRNA, and 100 µg/ml salmon sperm DNA. For hybridization 2×10^6 dpm (about 1 ng) of in vitro synthesized ^{32}P-labeled antisense RNA that had been mildly alkaline hydrolyzed was added directly to the sections. The hybridizations were performed by placing the sections into tightly sealed moist chambers for 12 hr at 50°C. After hybridizations the sections were washed in 50% formamide, 2× SSPE for 1 hr at 50°C with two changes of wash buffer. After an RNAase A treatment the sections were washed again (50% formamide, 0.1× SSPE; at 37°C), dehydrated, air-dried, and covered with a Kodak NTB2 emulsion (Kodak, Rochester, NY). Autoradiography was for 3–6 days. The sections were counter-stained with hematoxylin and eosin.

Cell Culture

The cell lines 7AD-7, 7AE-27, and XC (Svoboda, 1960) were grown in DMEM/HAMF12 (1:1) medium with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The day before harvesting the cells were washed twice with phosphate-buffered saline and induced under the following conditions in serum-free medium: glucocorticoid induction was for 24 hr in the presence of 10^{-6} M dexamethasone; cAMP induction was for 5 hr in the presence of 0.5 mM p-chlorophenylthio-cAMP (CPT-cAMP, Boehringer Mannheim). Double induction was accomplished by adding 10^{-6} M dexamethasone 24 hr before harvesting and 0.5 mM CPT-cAMP 5 hr before harvesting. The control was mock-induced with ethanol.

For transfections the calcium phosphate coprecipitation method (Graham and Van der Eb, 1973) was used. Reporter plasmid (1 pmol) and 0.5 pmol of either pHD or pHD-C/EBP were introduced into the hepatoma cell line FTO-2B together with an appropriate amount of plasmid carrier DNA. Four hours after addition of the precipitate, cells were stocked with 30% dimethyl sulfoxide in serum-free medium for 4 min. Incubation was then continued in serum-free medium. Preparation of extracts and CAT assays were performed as described (Boshart et al., 1990).

Animals

Mouse strains carrying the albino lethal deletions c^{3H} and c^{14CoS} (Gluecksohn-Waelsch, 1979; Russell et al., 1982) were used in the present experiments. Mice homozygous for these deletions die shortly after birth. Therefore, it is necessary to maintain them in the heterozygous state. Newborn homozygous deletion mice and heterozygotes as well as normal homozygotes were identified by the absence or presence of eye pigment.

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