Extinction of α_1 -antitrypsin gene expression in somatic cell hybrids: evidence for multiple controls

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Expression of the liver-specific α_1 -antitrypsin (α_1 AT) gene is extinguished in hepatoma/fibroblast hybrids. To define the mechanism of extinction, we identified DNA sequences involved in this process by transiently transfecting mutant α_1 AT promoters into parental and hybrid cells. The wild-type α_1 AT promoter (–554 to +44 bp) was highly expressed in rat hepatoma cells, but activity was 100-fold less in fibroblasts or cell hybrids. Mutations in this region failed to activate α_1 AT expression in nonhepatic cells, but mutations in the binding site for liver factor B1 (LF-B1) reduced hepatic-specific expression >100-fold. Furthermore, the hybrid cells failed to express LF-B1-binding activity and mRNA. This suggested that α_1 AT extinction in hybrids might be an indirect, lack-of-activation phenotype mediated primarily through repression of LF-B1. To test this possibility, we stably transfected an LF-B1 expression cassette into parental and hybrid cells and monitored expression of transfected and endogenous α_1 AT genes. Surprisingly, although constitutive LF-B1 expression could activate α_1 AT–CAT transgenes in these cells, it neither prevented nor reversed extinction of the chromosomal α_1 AT genes. We conclude that although extinction of the LF-B1 *trans*-activator accompanies α_1 AT extinction in cell hybrids, it does not play a causal role in this process.

[*Key Words*: Tissue-specific expression; gene regulation; hybrid cells]

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Mammalian development is a complex process that involves interdependent regulatory signals and responses. These processes culminate in the establishment of lineage-dependent patterns of transcription that are the basis of cell specialization. Most information about cell-specific transcription concerns positive regulation—the activation of cell-specific promoters by *trans*-acting protein factors (Maniatis et al. 1987). However, substantial evidence suggests that negative controls play important, but less-defined, roles in this process (Colantuoni et al. 1987; Nabel et al. 1988; Triputti et al. 1988; Yu et al. 1989; Vacher and Tilghman 1990). Somatic cell fusion experiments indicate that negative regulation is dominant in controlling phenotypic expression in intertypic hybrid cells (for review, see Gourdeau and Fournier 1990).

Extinction is a term that refers to the repression of tissue-specific gene activity that generally occurs in hybrids formed by fusing cells of different types (Davidson et al. 1966). Extinction is a transcriptional effect (Junker et al. 1988; Cereghini et al. 1990) that results in >1000-fold reductions in steady-state levels of tissue-specific mRNAs (Chin and Fournier 1987). Extinction of tissue-specific gene activity can be detected within hours of cell fusion (Thompson and Gelehrter 1971; Mével-Ninio and Weiss 1981), and most, if not all, cell-specific genes are

affected (Chin and Fournier 1987). This phenotype seems to have a specific genetic basis, as reexpression of extinguished genes is commonly observed in hybrid segregants that have eliminated parental chromosomes. Furthermore, monochromosomal hybrids retaining specific donor chromosomes can extinguish expression of particular cell-specific genes (Killary and Fournier 1984; Petit et al. 1986; Lem et al. 1988; Chin and Fournier 1989).

Little is known concerning the molecular mechanism of extinction. It has commonly been observed that tissue-specific promoters tend to be inactive when transfected into hybrid cells (Widen and Papaconstantinou 1987; Junker et al. 1988; McCormick et al. 1988; Triputti et al. 1988; Zaller et al. 1988). This suggests that extinction could be mediated through interactions at tissue-specific promoters and enhancers. Extinction of the genes encoding growth hormone (GH) and immunoglobulin heavy chain (IgH) has been associated with the loss of essential *trans*-activators, GHF-1 and Oct-2, in the hybrid cells (McCormick et al. 1988; Bergman et al. 1990). Furthermore, GH reexpression has been correlated with the reappearance of GHF-1 in hybrid segregants (McCormick et al. 1988). These results suggest that extinction might be an indirect effect mediated primarily through repression of *trans*-activators. However, a causal

relationship between extinction of a particular gene and loss of the relevant *trans*-activator has not yet been established.

The α_1 -antitrypsin (α_1 AT) gene is a useful model to study the molecular mechanism of extinction. The human α_1 AT gene contains a macrophage-specific promoter and a hepatocyte-specific promoter that are separated by ~2 kb of DNA. The proximal promoter is active in liver cells; it contains binding sites for two important *trans*-activators, LF-A1 and LF-B1 (De Simone et al. 1987; Li et al. 1988). Mutations in either of these sites reduce promoter activity in transfected human hepatoma cells (De Simone et al. 1987) and in rat liver nuclear extracts (Monaci et al. 1988). DNA sequences farther upstream have little effect on activity of the human α_1 AT promoter (De Simone et al. 1987; Shen et al. 1987). Thus, proximal promoter elements play dominant roles in establishing high-level liver-specific transcription. The murine α_1 AT promoter also requires LF-A1 and LF-B1 binding for maximal activity (Montgomery et al. 1990), but distal DNA elements may also be involved (Grayson et al. 1988).

LF-B1, also designated HNF-1, APF, or HP-1, activates expression of a number of liver genes. Transient transfection experiments have shown that LF-B1 binding is required for albumin (Herbomel et al. 1989) and α -fetoprotein (Feuerman et al. 1989) gene activity in cultured hepatoma cells. Other liver genes, including pyruvate kinase, α - and β -fibrinogen, transthyretin, aldolase B, and hepatic CYP2E1, show similar requirements for LF-B1 binding (for review, see Johnson 1990). Importantly, the LF-B1 *trans*-activator is not expressed in dedifferentiated hepatoma variants or extinguished cell hybrids (Baumhueter et al. 1988; Cereghini et al. 1988). The LF-B1 gene has been cloned, and *in vitro*-translated protein *trans*-activates LF-B1-dependent promoters *in vitro* (Frain et al. 1989; Nicosia et al. 1990). LF-B1 is expressed primarily in epithelial cells of endodermal and mesodermal origin (Baumhueter et al. 1990; De Simone et al. 1991). The LF-B1 protein contains a DNA-binding region composed of a dimerization domain (De Francesco et al. 1991), a region with weak homology to the POU domain of a number of eukaryotic transcription factors (Herr et al. 1988), and an unusual homeo domain with extra sequences in the region of the second helix (Nicosia et al. 1990).

To study the molecular mechanism of extinction, we transfected mutant α_1 AT promoters into various cell types and identified DNA sequences involved in this process. Although α_1 AT transgenes were extinguished in cell hybrids, we found no evidence that dominant-negative controls were involved. Rather, the inactivity of α_1 AT transgenes was primarily the result of a lack of LF-B1-mediated *trans*-activation, and the LF-B1 gene itself was extinguished in cell hybrids. However, constitutive LF-B1 expression in these cells neither prevented nor reversed α_1 AT extinction, although it was sufficient to *trans*-activate transiently transfected reporters. Thus, loss of *trans*-activators may not be a general mechanism of extinction.

Results

Endogenous α_1 -antitrypsin genes are extinguished in hepatoma/fibroblast hybrids

Rat hepatoma cells (FTO-2B) were fused with rat fibroblasts (Rat-1) or mouse embryo fibroblasts (MEFs), and polyclonal hybrid populations (FR and FM, respectively) were isolated. α_1 AT mRNA expression in the cells was assayed by RNA blot hybridization (Fig. 1). α_1 AT mRNA was readily detected in rat hepatoma cells but not in fibroblasts or cell hybrids. These results are consistent with previous observations of α_1 AT extinction in hepatoma cell hybrids (Pearson et al. 1983; Chin and Fournier 1987).

Identification of α_1 AT promoter elements involved in differential expression

α_1 AT promoter elements involved in differential expression were identified by transfecting various α_1 AT-chloramphenicol acetyltransferase transgenes (α_1 AT-CAT) into the different cell types. A plasmid containing α_1 AT promoter sequences from -554 to +44 bp driving CAT expression (Fig. 2A) was active in rat hepatoma cells but not in fibroblasts or cell hybrids (Fig. 2B). By normalizing to β -galactosidase activity expressed from a cotransfected cytomegalovirus (CMV)-*lac* reporter (Geballe and Mocarski 1988), we estimate that this α_1 AT promoter was 100- to 500-fold more active in FTO-2B cells than in fibroblasts or FM hybrids. Deleting α_1 AT promoter se-

Figure 1. The α_1 AT gene is extinguished in hepatoma/fibroblast hybrids. Cytoplasmic RNAs (10 μ g) from rat hepatoma cells (FTO-2B), MEFs or rat fibroblasts (Rat-1), and hepatoma/fibroblast hybrids (FM, FR) were size fractionated on 1.0% agarose gels, transferred to nylon membranes, and probed with a labeled 540-nucleotide mouse α_1 AT riboprobe. The filter was stripped and reprobed with an α -tubulin probe to control for RNA loading. Filters were exposed to film for 6–18 hr.

Figure 2. Activities of mutant α_1 AT promoters in parental and hybrid cells. (A) Regulatory sequences within the α_1 AT promoter. Binding sites for liver factors A1 (LF-A1) and B1 (LF-B1) are shown, as are regions with constitutive enhancer activity (En). The transcription start site is indicated by the arrow. (B) Analysis of 5' deletions. α_1 AT-CAT chimeric genes with 5' endpoints at -554, -400, -337, -200, -128, and -110 bp relative to the α_1 AT start site and a common 3' boundary at +44 bp were transfected into FTO-2B (narrowly hatched bar), MEF (open bar), and FM hybrid cells (widely hatched bar) by calcium phosphate coprecipitation. Plasmid CMV-lac was included to control for transfection efficiency. The cells were harvested after 40–48 hr and assayed for CAT and β -galactosidase activities. Corrected CAT activity for the largest construct (-554- to +44-bp α_1 AT-CAT) in FTO-2B cells was arbitrarily set at 100; the average value for this construct in FTO-2B cells was 320 pmoles/min per milligram of protein. Each level shown is an average of at least two trials. (C) Analysis of clustered point mutations. FTO-2B (hatched bar), Rat-1 (crosshatched bar), and FR (solid bar) hybrid cells were transfected with -261- to +44- bp α_1 AT-CAT constructs containing clustered point mutations, and CAT activities were calculated as described above. The activity of the wild-type -261 to +44 construct in FTO-2B cells was arbitrarily set at 100 (average activity, 250 pmoles/min per milligram of protein). Each value represents an average of two independent experiments. Promoter sequences protected from DNase I digestion by liver factors LF-A1 and LF-B1 and for ubiquitous factors LF-A2 and LF-B2 are shown (Monaci et al. 1988). Mutants PM1 and PM2 preclude LF-B1 binding, and EM3 and EM4 preclude LF-A1 binding (Hardon et al. 1988).

quences between -554 and -200 bp had little effect on promoter specificity, but α_1 AT-CAT transgenes with 5' boundaries at -128 or -110 bp were poorly expressed in all cell types. Thus, the proximal region of the α_1 AT promoter, which contains LF-A1- and LF-B1-binding sites (Fig. 2A), was required for high-level expression in rat hepatoma cells.

To define the relative contributions of LF-A1 and LF-B1 to α_1 AT promoter activation in these cells, we tested a series of α_1 AT-CAT chimeras with clustered point mutations in the region between -261 and +44 bp (Hardon et al. 1988). Mutations that precluded LF-A1 binding (EM3 and EM4) resulted in only a 5- to 10-fold decrease in α_1 AT promoter activity in transfected FTO-2B cells, but a mutant that prevented LF-B1 binding (PM1) was at least 100-fold less active than the wild-type promoter in these cells (Fig. 2C). These results indicate that LF-A1 plays a relatively minor role in activating the

α_1 AT promoter in rat hepatoma cells, and LF-B1 is the dominant *trans*-activator in these cells.

The 5'-deletion constructs and clustered point mutant α_1 AT promoters were also tested for activity in fibroblasts and cell hybrids (Fig. 2B,C, and data not shown). The -554- to +44-bp α_1 AT-CAT transgene was not expressed in either cell type, and none of the mutations we tested activated expression in these cells. Therefore, negative controls for repressing the α_1 AT promoter in non-hepatic cells were not apparent in these assays.

As an independent means to search for negative elements in the α_1 AT promoter, we compared the activities of an SV40 enhancer/promoter/CAT construct with that of a derivative with α_1 AT sequences inserted between the SV40 enhancer and promoter. In this construct, dominant-negative sequences might be expected to interfere with SV40 enhancer-mediated activation, as described previously for sequences in the IgH enhancer (Yu et al.

1989). However, the inserted α_1 AT sequences had no effect on SV40-driven CAT expression in either MEF or FM hybrid cells (Fig. 3).

These results do not exclude the possibility that negative elements might reside elsewhere in the α_1 AT gene or be covert in our assays. Nonetheless, the extinction of α_1 AT transgenes containing the proximal region of the promoter seems independent of negative control. These observations are most consistent with a model in which extinction of α_1 AT expression in fibroblasts and cell hybrids is the result of lack of *trans*-activation by liver-enriched factors.

Lack of α_1 AT promoter activity is associated with loss of trans-activating factors

The expression of LF-A1- and LF-B1-binding activities by parental and hybrid cells was monitored by incubating nuclear extracts with specific labeled oligonucleotides and resolving protein-DNA complexes by polyacrylamide gel electrophoresis. LF-B1-binding activity was readily detected in rat (FTO-2B) and human (HepG2) hepatoma cell nuclear extracts, but no binding activity was apparent in extracts from mouse (MEF) or rat fibroblasts (Rat-1) or from human HeLa cells (Fig. 4A). LF-B1-binding activity was absent or much reduced in FR and FM hybrid cell nuclear extracts.

LF-A1-binding activity showed a similar pattern of expression: LF-A1 was expressed in hepatoma cells but not in fibroblasts or cell hybrids (Fig. 4B). However, LF-A1 was differentially expressed in human and rat hepatoma cells, with HepG2 expressing significantly more LF-A1-binding activity than FTO-2B. This may explain why LF-A1 apparently plays a minor role in α_1 AT promoter activation in rat hepatoma cells (see above).

These results indicate that both LF-A1- and LF-B1-binding activities are extinguished in hepatoma cell hybrids. In contrast, the ubiquitous *trans*-activator Oct-1 was expressed in all cell lines tested (Fig. 4C). Furthermore, the specificity of the LF-A1, LF-B1, and Oct-1 pro-

tein-DNA complexes was established by incubating each probe/extract mixture with a 50-fold molar excess of specific or nonspecific unlabeled oligonucleotide competitor (Fig. 4D-F). LF-B1-specific binding was also unaffected by heating the nuclear extracts to 65°C or by the presence of 9 mM Mg^{2+} , properties characteristic of bona fide LF-B1 binding (Cereghini et al. 1988).

Cloned LF-B1 trans-activates transfected α_1 AT promoters in nonhepatic cells

If extinction of α_1 AT gene expression in cell hybrids were primarily the result of loss of LF-B1 *trans*-activation, forced expression of LF-B1 in the cells should then activate the α_1 AT promoter. As a first test of this model, we cotransfected cells with an α_1 AT-CAT reporter together with either an LF-B1 expression plasmid (pRSVB1) or vector (pUC18) DNA (Fig. 5). FTO-2B cells, which express endogenous LF-B1, expressed the α_1 AT-CAT transgene at high levels with or without exogenous LF-B1. In contrast, FR hybrid or Rat-1 fibroblast recipient cells expressed the α_1 AT-CAT reporter only in the presence of pRSVB1; *trans*-activation was 23- and 66-fold relative to vector-cotransfected cells. This *trans*-activation required LF-B1 binding, because the PM1 mutant α_1 AT promoter could not be *trans*-activated. A related promoter mutant that did bind LF-B1 (PM3) was *trans*-activated efficiently. These results indicate that *trans*-activation of the α_1 AT-CAT reporter was a specific effect of LF-B1 mediated through the LF-B1-binding site. Interestingly, levels of α_1 AT-CAT reporter gene activity in nonhepatic cells cotransfected with pRSVB1 were only 5- to 10-fold less than those of α_1 AT-CAT-transfected rat hepatoma cells. On the basis of our analyses of mutant α_1 AT promoters (above), this phenotype would be expected for cells expressing LF-B1 but not LF-A1. Thus, forced expression of LF-B1 in fibroblasts or hybrid cells restored activity of a transiently cotransfected α_1 AT reporter.

Figure 3. Activities of a two-enhancer construct. MEF (hatched bar) and FM (stippled bar) hybrid cells were transfected with plasmids in which CAT gene expression was driven from the SV40 minimal promoter (SV-CAT), the SV40 enhancer and promoter (SVEN-SV-CAT), or a plasmid with α_1 AT sequences (–137 to –37 bp) inserted between the SV40 enhancer and promoter (SVEN-AT-SV-CAT). The cells were harvested after 40–48 hr, and CAT and β -galactosidase activities were determined. The activity of pSV2CAT (SVEN-SV-CAT) in MEF cells was arbitrarily set at 100.

Figure 4. Hybrid cells lack LF-A1- and LF-B1-binding activities. Nuclear extracts were incubated with end-labeled oligonucleotides containing binding sites for LF-B1 (A,D), LF-A1 (B,E), or Oct-1 (C,F), and specific protein–DNA complexes (arrows) were resolved by electrophoresis. A–C show binding activities in nuclear extracts (preheated to 65°C for 5 min in A) from human hepatoma cells (HepG2), human HeLa cells, rat hepatoma cells (FTO-2B), mouse embryo fibroblasts (MEF), FTO-2B × MEF hybrid cells (FM), Rat-1 fibroblasts, and FTO-2B × Rat-1 hybrids (FR). C–E show binding activities of rat hepatoma cell (FTO-2B) nuclear extracts in the presence of a 50-fold excess of unlabeled competitor oligonucleotides containing binding sites for LF-B1 (B1), LF-B2 (B2), LF-A2 (A2), LF-A1 (A1), or Oct-1. (–) No competitor added. LF-B1-specific binding (D) was unaffected by heating the extracts to 65°C or by the presence of 9 mM Mg²⁺. (F) The position of free, uncomplexed oligonucleotides.

Constitutive expression of cloned LF-B1 does not reverse α_1 AT extinction

As constitutive LF-B1 expression in extinguished cells allowed *trans*-activation of transiently transfected α_1 AT reporters, we then asked whether forced LF-B1 expression could reverse extinction of their chromosomal α_1 AT genes. Stable Rat-1 and FR transfectants expressing cDNA cassettes encoding LF-B1 or DNA-binding mutant SM6 (Fig. 6A) were prepared (Fig. 6B) and assayed for expression of LF-B1 and α_1 AT mRNAs. Substantial levels of LF-B1 or SM6 mRNA were expressed in each stably transfected cell population (Fig. 6C). Transfectant subclones from each population were also analyzed: About half of them expressed the transfected LF-B1 or

SM6 cDNA (data not shown). LF-B1 mRNA levels were somewhat higher in Rat-1 transfectants than in FR transfectants, but all of the transfected cell populations expressed substantial amounts of LF-B1 mRNA. Furthermore, these cells expressed functional LF-B1, because the LF-B1 transfectants (Rnb1 and FRnb1) efficiently *trans*-activated a transiently transfected α_1 AT–CAT reporter (Fig. 7). In contrast, SM6-expressing cells failed to *trans*-activate, but transient cotransfection with pRSVB1 increased α_1 AT–CAT expression >50-fold (Fig. 7). This was a promoter-specific effect, as a transfected herpesvirus thymidine kinase (TK) promoter was expressed at similar levels in LF-B1- and SM6-expressing cells (data not shown). Therefore, the stable fibroblast and hybrid cell transfectants expressed functional LF-B1 at levels

Figure 5. Transfected α_1 AT promoters are *trans*-activated by cloned LF-B1. FTO-2B (hatched bar), Rat-1 (stippled bar), and FR (solid bar) hybrid cells were cotransfected with α_1 AT-CAT plasmids with or without pRSVB1 (containing LF-B1 cDNA under control of the RSV LTR). pCMV-lac was included as an internal control. β -Galactosidase-corrected CAT activities were normalized to the activity of (-261 to +44 bp) α_1 -CAT in FTO-2B cells, which was set at 100. The PM1 mutant promoter is unable to bind LF-B1 (De Simone et al. 1987). The PM3 mutant promoter was as active as the wild-type promoter in FTO-2B cells. Numbers above the bars indicate the fold increase in α_1 AT-CAT promoter activity when pRSVB1 was cotransfected. Each value is an average of two independent experiments.

sufficient to fully *trans*-activate a transiently transfected α_1 AT reporter. However, neither fibroblast (Rnb1) nor hybrid (FRnb1) LF-B1-transfectants expressed detectable mRNA from their chromosomal α_1 AT genes (Fig. 6C). Titration experiments indicated that blot hybridizations performed under these conditions could readily detect α_1 AT mRNA at levels $\frac{1}{300}$ those of FTO-2B cells (Fig. 6D), so that gene activation would have been apparent even if it occurred in only a fraction of the hybrid cells. Thus, forced expression of LF-B1 was not sufficient to reactivate extinguished α_1 AT genes in fibroblasts or cell hybrids.

Constitutive expression of cloned LF-B1 does not prevent α_1 AT extinction

The inability of cloned LF-B1 to reverse α_1 AT extinction in hybrids might have been due to the fact that the α_1 AT genes had been extinguished for many cell generations and were refractory to reactivation. Therefore, we determined whether continuous expression of LF-B1 throughout the life history of a hybrid might prevent α_1 AT extinction. Rat-1 fibroblasts constitutively expressing LF-

B1 (Rnb1) or SM6 (RnSM) were fused with FTO-2B rat hepatoma cells, and the resulting hybrid populations (Rnb1F and RnSMF, Fig. 6B) were assayed for LF-B1 and α_1 AT mRNA expression by RNA blot hybridization. LF-B1 mRNA levels in the hybrids were reduced relative to parental Rnb1 or RnSM cells (Fig. 6C), and analyses of hybrid subclones indicated that about half of them expressed transfected LF-B1 (Fig. 6E). However, none of the LF-B1-expressing hybrids or subclones expressed detectable α_1 AT mRNA from their chromosomal α_1 AT genes (Fig. 6C,E), despite the fact that LF-B1 was expressed at levels sufficient to *trans*-activate transiently transfected α_1 AT reporters (Fig. 7). As functional LF-B1 was expressed before, during, and after fusion in these cells, we conclude that constitutive LF-B1 expression was not sufficient to prevent α_1 AT extinction. Therefore, α_1 AT extinction in these cells is not simply the result of a lack of functional LF-B1.

Discussion

Extinction in somatic cell hybrids has been studied for more than 25 years, but little is known about molecular events that direct this process. In general, tissue-specific promoters are inactive in cell hybrids (Petit et al. 1986; Widen and Papaconstantinou 1987; Junker et al. 1988; McCormick et al. 1988; Tripputi et al. 1988; Zaller et al. 1988; Yu et al. 1989; Bergman et al. 1990), but the DNA sequences that mediate these effects are largely unknown. In the few cases in which specific DNA targets have been defined, they mediate lack-of-activation phenotypes that may not be directly involved in extinction. For example, α_1 AT transgenes were poorly expressed in our hepatoma hybrids primarily because of a lack of LF-B1-mediated *trans*-activation. This is reminiscent of results from other hybrid cell systems, notably the GH gene in pituitary cell/fibroblast hybrids (McCormick et al. 1988) and the IgH gene in B-cell/fibroblast hybrids (Junker et al. 1988; Bergman et al. 1990). In all of these cases, extinction is accompanied by loss of specific *trans*-activators, and it behaves as a lack-of-activation phenotype that maps to the relevant binding sites. These results suggest a model in which extinction is not true repression but, rather, reflects lack of activation. However, our data do not support this model in its simplest form. In particular, we find that although α_1 AT extinction may be associated with loss of its major *trans*-activator, it is certainly not caused by it.

The recent molecular cloning of tissue-specific extinguisher-1 (TSE1) (Jones et al. 1991), a locus that down-regulates the expression of a number of liver genes in cell hybrids, provides another example of a transcriptional lack-of-activation phenotype in hybrid cells. The TSE1 gene product is an inhibitory subunit of protein kinase A: Its overexpression results in hypophosphorylation of a specific *trans*-activator (CREB) and down-regulation of target genes. Whether this dramatic lack-of-activation phenotype plays a central role in extinction is not clear, as TSE1-responsive genes can be repressed by other factors (R.E.K. Fournier, unpubl.). Therefore, the precise

roles that lack-of-activation phenotypes play in extinction have yet to be defined.

It is noteworthy that forced expression of LF-B1 in nonexpressing cells could activate expression of transiently transfected α_1 AT-CAT reporters, but it had no demonstrable effect on expression of the chromosomal α_1 AT genes. Thus, LF-B1 repression may be the reason that transfected α_1 AT reporter genes are poorly expressed in hepatoma hybrids, but it is clearly not a dominant force in extinction. This suggests that transfected genes lack sequences and/or structures that allow them to fully respond to extinction, so that *trans*-activation appears to be dominant. Similar results have been obtained in *in vitro* transcription reactions. For example, the α_1 AT promoter is fully active in mixed hepatoma plus fibroblast nuclear extracts (G.A. Bulla, unpubl.), and other liver genes show similar patterns of expression *in vitro* (Gorski et al. 1986; Lichtsteiner and Schibler 1989). Thus, extinction studies in these simpler systems may be uninformative, perhaps even misleading, because the true phenotype is not reconstituted. Our results also show that the coordinate regulation of *trans*-activators and target genes that is often apparent in cell hybrids does not necessarily reflect causality. Previous studies had suggested this possibility. For example, although loss of Oct-2-binding activity tends to accompany IgH extinction in B-cell/fibroblast hybrids, (Bergman et al. 1990), the Oct-2 and IgH extinction phenotypes are dissociable in T/B-cell hybrids (Yu et al. 1989).

It is unlikely that our inability to activate the endogenous α_1 AT genes of LF-B1-expressing hybrids was the result of irreversible inactivation of the genes in these cells. Many studies have shown that extinguished genes can be reexpressed in hybrid segregants: Extinction is reversible (for review, see Gourdeau and Fournier 1990). Furthermore, hepatoma hybrids that do not extinguish hepatic functions frequently activate expression of liver genes encoded by their nonhepatic parent (Peterson and Weiss 1972; Peterson et al. 1985; Barton and Franke 1987). Thus, silent mammalian genes are not irreversibly repressed; in the absence of extinction, activation can

occur. Furthermore, our results show that forced expression of LF-B1 could not prevent α_1 AT gene extinction. Therefore, the first critical tests of the hypothesis that extinction is mediated primarily by the loss of *trans*-activators has failed to provide support for that model in its simplest form.

LF-B1 is the major *trans*-activator of the α_1 AT promoter in rat hepatoma cells; LF-A1, which is expressed at low levels, plays a relatively minor role. Accordingly, hybrid and fibroblast transfectants that constitutively expressed LF-B1 were able to *trans*-activate α_1 AT-CAT reporters to high levels, typically 20–25% those of rat hepatoma cells. LF-A1 was not expressed in these transfectants, so this result was consistent with our prior observation that LF-A1-binding site mutations reduced promoter strength only 5- to 10-fold in rat hepatoma cells. Both observations suggest that LF-A1 and LF-B1 *trans*-activations at the proximal α_1 AT promoter are largely independent events. As LF-B1 was the major *trans*-activator in this system, we would expect its effects, if any, on α_1 AT extinction to have been most readily apparent. Our inability to detect such effects suggests that lack of LF-B1-mediated *trans*-activation is not the immediate cause of α_1 AT extinction. However, we cannot exclude the possibility that constitutive expression of both LF-A1 and LF-B1 would prevent extinction. This appears unlikely in view of the apparently independent functions of LF-A1 and LF-B1 at the proximal promoter, but it is possible that they could interact in very different ways at other regulatory sites.

The developmental regulation of liver-specific gene expression is complex, as transcriptional activities of different liver genes change distinctly during embryogenesis. For example, the α -fetoprotein gene is active during fetal life but largely inactive after birth (Hammer et al. 1987). The α_1 AT and albumin genes are activated early and become progressively more highly expressed, whereas other liver functions are activated perinatally. The mechanisms that control these temporal changes in gene activity are not known, but tissue-specific *trans*-activators and repressors are likely to be involved. Be-

Figure 6. LF-B1 and α_1 AT expression in stably transfected clones. (A) Plasmid constructs used to generate Rat-1-derived (Rn series) and FR hybrid-derived (FRn series) LF-B1 transfectants. In each plasmid, LF-B1 cDNA expression is driven from the RSV LTR, and the neomycin phosphotransferase gene expression is driven by the SV40 early promoter. The LF-B1 mutant SM6 contains a double frameshift mutation that prevents binding of its product to the LF-B1-binding site (Nicosia et al. 1990). (B) Generation of cell lines constitutively expressing cloned LF-B1. Cells were transfected by electroporation with plasmids linearized at a restriction site downstream from the SV40 polyadenylation signal 3' of the LF-B1 cDNA. Rnb1/RnSM and FRnb1/FRnSM polyclones were selected in media containing G418. The Rnb1 and RnSM populations were subsequently fused with FTO-2B cells, and hybrids were selected in media containing G418 plus ouabain. These hybrid lines are designated Rnb1F and RnSMF, respectively. All transfectants were pooled. (C) LF-B1 mRNA expression in stably transfected cells. Cytoplasmic RNA (10 μ g) was size fractionated on a 1% agarose gel, transferred to a nylon filter, and hybridized with a random-primed, LF-B1-specific DNA probe. The filter was subsequently stripped and reprobed with an α_1 AT riboprobe followed by an α -tubulin probe. Cell lines Rnb1 and FRnb1 are Rat-1 and FR transfectant polyclones, respectively, expressing cloned LF-B1. Lines RnSM and FRnSM are Rat-1 and FR transfectant polyclones, respectively, expressing the SM6 mutant of LF-B1, which does not bind to DNA. (D) Sensitivity of blot hybridization for α_1 AT mRNA detection. RNAs from the indicated cell lines were fractionated on a 1% agarose gel, transferred to a nylon filter, and hybridized with labeled LF-B1, α_1 AT, and α -tubulin probes as described in Materials and Methods. Serial twofold dilutions of FTO-2B cytoplasmic RNA from 10 to 0.035 μ g were included. (E) LF-B1, α_1 AT, and α -tubulin mRNA expression in clonal Rnb1F transfectants. Cytoplasmic RNA blots were hybridized with labeled probes as described above. LF-B1 mRNA expression in 15 Rnb1F hybrid subclones and a single RnSMF clone was compared with mRNA expression of FTO-2B cells and the RnSMF polyclone.

Figure 7. Stable LF-B1 transfectants *trans*-activate a transiently introduced α_1 AT-CAT reporter. Stable fibroblast (RnSM, Rnb1) and hybrid (FRnSM, FRnb1, RnSMF, Rnb1F) cell transfectants expressing cloned LF-B1 were transiently transfected with an (–261 to +44 bp) α_1 AT-CAT reporter plus CMV-*lac* with or without a cotransfected RSV-LF-B1 expression plasmid. Cells were harvested after 48 hr, and extracts were assayed for CAT and β -galactosidase activities. Activation of the α_1 AT-CAT reporter under various conditions is shown. Fold activation of CAT activity upon cotransfection with pRSVB1 is shown for each cell line.

cause silent α_1 AT genes can be activated in hepatoma/fetal fibroblast hybrids (Barton and Francke 1987), differentiated liver cells can supply nonexpressing cells with *trans*-acting factors required for gene activation. However, our experiments suggest that lack of activation is not sufficient to explain gene inactivity in nonexpressing cells, and dominant-negative controls are likely to be involved. Hybrid cells provide one means of studying these regulatory interactions, which seem less apparent in simpler genetic and biochemical tests.

Materials and methods

Cell lines and culture conditions

Rat hepatoma FTO-2B cells are a ouabain-resistant, TK-deficient (Oua^r, TK[–]) derivative of H4IIEC3 (Killary and Fournier 1984). MEFs were prepared from 12- to 14-day C57BL/6J embryos according to standard techniques (Kozak et al. 1975). Rat1 is a line of SV40-transformed rat embryo fibroblasts (Botchan et al. 1976). Somatic cell hybrids were generated by PEG-mediated fusion (Killary and Fournier 1984) followed by selection in medium containing hypoxanthine/aminopterin/thymidine (HAT) plus 3 mM ouabain. G418 (500 μ g/ml) was included in fusions involving *neo*-transfected parental cells.

The cells were maintained in 1:1 Ham's F12/Dulbecco's modified Eagle medium containing 10% fetal bovine serum (GIBCO). Hybrid lines were maintained in selective medium, and antibiotics were not employed. All cell lines were free of mycoplasma as judged by staining with Hoechst 33258 (Chen 1977).

Plasmid constructs

The recombinant plasmids containing α_1 AT promoter fragments fused to CAT have been described (De Simone et al.

1987). The α_1 AT-CAT inserts of the original plasmids were isolated after *KpnI*/*HindIII* digestion and inserted into the *KpnI*/*HindIII* sites of pUC19CATt. pUC19CATt was prepared by inserting a trimer of the SV40 termination/polyadenylation sequence from pUC.A.1.5 (Maxwell et al. 1989) into the blunted *SacI* site of pUC19CAT2 (De Simone et al. 1987). This placed SV40 termination sequences immediately upstream of α_1 AT promoter sequences, preventing readthrough transcription (data not shown).

Plasmid pCMVlac (pQ176; Geballe and Mocarski 1988) contains the CMV immediate early promoter fused to the β -galactosidase gene. pTKCAT was constructed by inserting the herpes simplex virus TK promoter (*HindIII*/*PvuII* fragment) from pTKCAT into the *HindIII*/*PvuII* site of pSV0tCAT (Jones and Whitlock 1990). This placed TK-CAT sequences immediately downstream of a dimer of the SV40 polyadenylation/termination signal. Plasmid pSV137SV-CAT (De Simone and Cortese 1989) contains the proximal α_1 AT promoter (–137 to –37 bp) inserted between the SV40 enhancer and promoter in pSV2CAT (Gorman et al. 1982).

Plasmid pKOneoB1 contains the Rous sarcoma virus long terminal repeat (RSV LTR) driving expression of LF-B1 cDNA (Fraen et al. 1989) and the neomycin phosphotransferase gene driven from the SV40 early promoter. It was constructed by inserting an RSV-LF-B1 cassette (*SfiI* fragment of pRSVB 1) into the blunted *BamHI* site of pKOneo (from K. Yamamoto, University of California, San Francisco), with neo and LF-B1 in the same transcriptional orientation. pKOneoSM was constructed in a similar manner, but it contains two frameshift mutations in the LF-B1-coding sequence, which inactivate the DNA-binding domain (Nicosia et al. 1990).

DNA transfections

Transient transfections were performed by calcium phosphate coprecipitation (Wigler et al. 1979). Each transfection mixture contained 10 μ g of α_1 AT-CAT plasmid DNA, 1 μ g of pCMV-lac DNA, and 10 μ g pUC18 carrier DNA. The coprecipitate was incubated with recipient cells for 6–8 hr, the cells were washed three times with HEPES buffer, and fresh medium was added. After 36–48 hr, the cells were washed three times with PBS, incubated for 5 min in 150 mM NaCl, 1 mM EDTA, and 40 mM Tris (pH 7.4), and harvested by scraping. The cells were pelleted, suspended in 0.25 M Tris (pH 7.4), freeze-thawed three times, and centrifuged at 12,000g for 10 min at 4°C. Supernatants were assayed for protein concentration (Bradford 1976) and β -galactosidase activity by incubation with 4-methylumbelliferyl- β -galactosidase (MUG; Sigma Chemical Company) as described (Geballe and Mocarski 1988). Fluorescence was measured using a Microfluorimeter (Dynatech Industries Inc.). The remaining cell lysate was heated to 65°C for 5 min to inactivate deacetylase activity (Crabb and Dixon 1987) and centrifuged at 12,000g for 5 min, and the supernatant was assayed for CAT activity. Typically, 5–10 μ g of extract was added to a solution containing 0.25 M Tris (pH 7.4), 0.25 μ Ci [¹⁴C]chloramphenicol (New England Nuclear), and 2 mM acetyl-CoA (Pharmacia). Reactions were incubated for 1–7 hr at 37°C with the addition of fresh acetyl-CoA every 2 hr, followed by extraction with ethyl acetate and analysis by thin-layer chromatography (Gorman et al. 1982). Percent [¹⁴C]chloramphenicol conversion was quantitated by liquid scintillation counting, and values were normalized to β -galactosidase activities of the same samples.

Stable transfections were performed by electroporation (Chu et al. 1987) using a Bio-Rad Gene Pulser and Capacitance Extender. Exponentially growing cells were trypsinized, sus-

pended to 1.2×10^7 /ml in ice-cold PBS with 20 μ g of linearized plasmid DNA, and pulsed with 960 μ F at 300 V. The cells were harvested and replated in nonselective medium. G418 selective agent (500 μ g/ml) was added after 48 hr.

Gel-shift assays

Nuclear extracts were prepared (Shapiro et al. 1988) and incubated with 32 P-end-labeled, double-stranded oligonucleotides: LF-B1, CCTTGGTTAATATTCACC; LF-A1, CAGCCAGTGG-ACTAGCCCCCTGTTGCT; Oct-1, GGGGGTAATTTGCAT-TTCTAAGGG. Unlabeled competitor oligonucleotides were included in some reactions, including an LF-A2-specific oligonucleotide [GATCCTGTTTGCTCCTCCGATAAC]. Binding reactions included 5–10 μ g of nuclear extract in 7.5% glycerol, 60 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.75 mM dithiothreitol, 0.5% aprotinin, 2 mM benzimidazole, 0.3 μ g/ml of leupeptin, 1 μ g/ml of antipain, 1 mM PMSF, 2 μ g of poly[d(I-C)], and 25 mM HEPES (pH 7.6). The samples were incubated at room temperature for 30 min, loaded onto 4% polyacrylamide gels, and run at 15 V/cm for 3–4 hr. Gels were dried and exposed to film for 1–5 days.

Blot hybridizations

Cytoplasmic RNA was extracted from nearly confluent cultures as described (Favaloro et al. 1980). RNA (5–10 μ g) was denatured in 50% formamide at 65°C for 15 min and loaded onto a 1% agarose-formaldehyde gel (Maniatis et al. 1982). Gels were run at 7 V/cm for 4 hr, and the RNA was transferred to nylon membranes (Zetabind, Cuno, Inc.) overnight. The blots were prehybridized for ~1 hr, and labeled probe was added. To detect α_1 AT mRNA, a 540-nucleotide radiolabeled antisense RNA probe corresponding to the 3' end of mouse α_1 AT mRNA was used (from H. Hastie, Edinburgh, UK). Filters were hybridized at 65°C in 50% formamide, 5 $\times$ SSPE (1 $\times$ SSPE = 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate at pH 7.4), 1% SDS, 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), and 10 μ g/ml each of poly(A) and poly(C) (Pharmacia). After incubation overnight, the filters were washed twice in 2 $\times$ SSC (1 $\times$ SSC = 150 mM NaCl, 15 mM sodium citrate at pH 7.0), 0.1% SDS, at room temperature, followed by two 30-min washes in 0.1 $\times$ SSC, 0.1% SDS, at 65°C. The LF-B1 probe was a random-primed 3.6-kb *Sfi*I fragment of pRSVB1. The human α -tubulin probe was a random-primed 1.6-kb cDNA fragment from p κ al (Cowan et al. 1983). LF-B1 and α -tubulin probes were incubated with filters in hybridization solution (50% formamide, 1% bovine serum albumin, 5% SDS, 1 mM EDTA, 0.5 M NaHPO₄, 0.08 mg/ml of yeast tRNA at pH 7.2) overnight, followed by two 15-min washes in 2 $\times$ SSC, 0.1% SDS, and two 30-min washes at 56°C in 0.1 $\times$ SSC, 0.1% SDS. Filters were exposed to film for 1–3 days.

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