Extinction of $\alpha_1$-antitrypsin gene expression in somatic cell hybrids: evidence for multiple controls

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Expression of the liver-specific $\alpha_1$-antitrypsin ($\alpha_1$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 $\alpha_1$AT promoters into parental and hybrid cells. The wild-type $\alpha_1$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 $\alpha_1$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 $\alpha_1$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 $\alpha_1$AT genes. Surprisingly, although constitutive LF-B1 expression could activate $\alpha_1$AT–CAT transgenes in these cells, it neither prevented nor reversed extinction of the chromosomal $\alpha_1$AT genes. We conclude that although extinction of the LF-B1 trans-activator accompanies $\alpha_1$AT extinction in cell hybrids, it does not play a causal role in this process.

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

Received September 5, 1991; revised version accepted December 10, 1991.

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 α1-antitrypsin (α1,AT) gene is a useful model to study the molecular mechanism of extinction. The human α1,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 α1,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 α1,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 α1,AT promoters into various cell types and identified DNA sequences involved in this process. Although α1,AT transgenes were extinguished in cell hybrids, we found no evidence that dominant-negative controls were involved. Rather, the inactivity of α1,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 α1,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 α1-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. α1,AT mRNA expression in the cells was assayed by RNA blot hybridization (Fig. 1). α1,AT mRNA was readily detected in rat hepatoma cells but not in fibroblasts or cell hybrids. These results are consistent with previous observations of α1,AT extinction in hepatoma cell hybrids [Pearson et al. 1983; Chin and Fournier 1987].

Identification of α1,AT promoter elements involved in differential expression

α1,AT promoter elements involved in differential expression were identified by transfecting various α1,AT–chloramphenicol acetyltransferase transgenes [α1,AT–CAT] into the different cell types. A plasmid containing α1,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 α1,AT promoter was 100- to 500-fold more active in FTO-2B cells than in fibroblasts or FM hybrids. Deleting α1,AT promoter se-
Figure 2. Activities of mutant $\alpha_1$AT promoters in parental and hybrid cells. (A) Regulatory sequences within the $\alpha_1$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. $\alpha_1$AT–CAT chimeric genes with 5′ endpoints at -554, -400, -337, -200, -128, and -110 bp relative to the $\alpha_1$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 $\beta$-galactosidase activities. Corrected CAT activity for the largest construct (-554- to +44-bp $\alpha_1$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 -1-44-bp $\alpha_1$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-Al and LF-Bl and for ubiquitous factors LF-A2 and LF-B2 are shown (Monaci et al. 1988). Mutants PM1 and PM2 preclude LF-Bl binding, and EM3 and EM4 preclude LF-A1 binding (Hardon et al. 1988).

sequences between -554 and -200 bp had little effect on promoter specificity, but $\alpha_1$AT–CAT transgenes with 5′ boundaries at -128 or -110 bp were poorly expressed in all cell types. Thus, the proximal region of the $\alpha_1$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 $\alpha_1$AT promoter activation in these cells, we tested a series of $\alpha_1$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 $\alpha_1$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 $\alpha_1$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 $\alpha_1$AT promoters were also tested for activity in fibroblasts and cell hybrids (Fig. 2B,C, and data not shown). The -554- to +44-bp $\alpha_1$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 $\alpha_1$AT promoter in non-hepatic cells were not apparent in these assays.

As an independent means to search for negative elements in the $\alpha_1$AT promoter, we compared the activities of an SV40 enhancer/promoter/CAT construct with that of a derivative with $\alpha_1$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.
However, the inserted α1-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 α1-AT gene or be covert in our assays. Nonetheless, the extinction of α1-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 α1-AT expression in fibroblasts and cell hybrids is the result of lack of trans-activation by liver-enriched factors.

**Lack of α1-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 α1-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 protein–DNA complexes was established by incubating each probe/extract mixture with a 50-fold molar excess of specific or nonspecific unlabelled 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 Mg2+, properties characteristic of bona fide LF-B1 binding (Cereghini et al. 1988).

**Cloned LF-B1 trans-activates transfected α1-AT promoters in nonhepatic cells**

If extinction of α1-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 α1-AT promoter. As a first test of this model, we cotransfected cells with an α1-AT–CAT reporter together with either an LF-B1 expression plasmid (pRSVBl) or vector (pUC18) DNA (Fig. 5). FTO-2B cells, which express endogenous LF-B1, expressed the α1-AT–CAT transgene at high levels with or without exogenous LF-B1. In contrast, FR hybrid or Rat-1 fibroblast recipient cells expressed the α1-AT–CAT reporter only in the presence of pRSVBl; trans-activation was 23- and 66-fold relative to vector-cotransfected cells. This trans-activation required LF-B1 binding, because the FM1 mutant α1-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 α1-AT–CAT reporter was a specific effect of LF-B1 mediated through the LF-B1-binding site. Interestingly, levels of α1-AT–CAT reporter gene activity in nonhepatic cells cotransfected with pRSVBl were only 5- to 10-fold less than those of α1-AT–CAT-transfected rat hepatoma cells. On the basis of our analyses of mutant α1-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 α1-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 α1-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.
Constitutive expression of cloned LF-B1 does not reverse α1AT extinction

As constitutive LF-B1 expression in extinguished cells allowed trans-activation of transiently transfected α1AT reporters, we then asked whether forced LF-B1 expression could reverse extinction of their chromosomal α1AT 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 α1AT 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 (Rnbl and FRnbl) efficiently trans-activated a transiently transfected α1AT-CAT reporter (Fig. 7). In contrast, SM6-expressing cells failed to trans-activate, but transient cotransfection with pRSVB1 increased α1AT-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
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, α1AT 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 α1AT 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
Figure 6. (See facing page for legend).
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 \( \alpha_1 \)AT–CAT reporters, but it had no demonstrable effect on expression of the chromosomal \( \alpha_1 \)AT genes. Thus, LF-B1 repression may be the reason that transfected \( \alpha_1 \)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 \( \alpha_1 \)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 \( \alpha_1 \)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 \( \alpha_1 \)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 \( \alpha_1 \)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 \( \alpha_1 \)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 \( \alpha_1 \)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 \( \alpha_1 \)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 \( \alpha_1 \)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 distinctively during embryogenesis. For example, the \( \alpha \)-fetoprotein gene is active during fetal life but largely inactive after birth (Hammer et al. 1987). The \( \alpha_1 \)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.

**Figure 6.** LF-B1 and \( \alpha_1 \)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. Rnbl/RnSM and FRnbl/FRnSM polyclones were selected in media containing G418. The Rnbl 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 RnblF and RnSMF, respectively. All transfectants were pooled. (C) LF-B1 mRNA expression in stably transfected cells. Cytoplasmic RNA (10 \( \mu \)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 \( \alpha_1 \)AT riboprobe followed by an \( \alpha \)-tubulin probe. Cell lines RnblF and FRnbl 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 \( \alpha_1 \)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, \( \alpha_1 \)AT, and \( \alpha \)-tubulin probes as described in Materials and methods. Serial twofold dilutions of FTO-2B cytoplasmic RNA from 10 to 0.035 \( \mu \)g were included. (E) LF-B1, \( \alpha_1 \)AT, and \( \alpha \)-tubulin mRNA expression in cloned RnblF transfectants. Cytoplasmic RNA blots were hybridized with labeled probes as described above. LF-B1 mRNA expression in 15 RnblF hybrid subclones and a single RnSMF clone was compared with mRNA expression of FTO-2B cells and the RnSMF polyclone.
cause silent αAT 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 an ouabain-resistant, TK-deficient [Ouf, TK-] derivative of H4IEC3 [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) and 10% fetal bovine serum (FBS). Transient transfections were performed by calcium phosphate coprecipitation [Wigler et al. 1979]. Each transfection mixture contained 10 μg of αAT–CAT plasmid DNA, 1 μg of pCMV–lac DNA, and 10 μg 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, resuspended in 0.25 mM 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 deacteylase activity [Crabb and Dixon 1987] and centrifuged at 12,000g for 5 min, and the supernatant was assayed for CAT activity. Typically, 5–10 μg of extract was added to a solution containing 0.25 mM Tris (pH 7.4), 0.25 mM 14Cchloramphenicol [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 14Cchloramphenicol 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-

Figure 7. Stable LF-B1 transfectants trans-activate a transiently introduced αAT–CAT reporter. Stable fibroblast (RnSM, Rnbl) and hybrid (FRnSM, FRnbl, RnSMF, RnblF) cell transfectants expressing cloned LF-B1 were transiently transfected with an [-261 to +44 bp] αAT–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 αAT–CAT reporter under various conditions is shown. Fold activation of CAT activity upon cotransfection with pRSVB1 is shown for each cell line.

Plasmid constructs

The recombinant plasmids containing αAT promoter fragments fused to CAT have been described [De Simone et al. 1987]. The αAT–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 blunt-ended SacI site of pUC19CAT2 (De Simone et al. 1987). This placed SV40 termination sequences immediately upstream of αAT 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 Costese 1989] contains the proximal αAT 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 (R5V LTR) driving expression of LF-B1 cDNA [Frain 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 blunt-ended 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 μg of αAT–CAT plasmid DNA, 1 μg of pCMV–lac DNA, and 10 μg 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, resuspended in 0.25 mM 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 deacteylase activity [Crabb and Dixon 1987] and centrifuged at 12,000g for 5 min, and the supernatant was assayed for CAT activity. Typically, 5–10 μg of extract was added to a solution containing 0.25 mM Tris (pH 7.4), 0.25 mM 14Cchloramphenicol [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 14Cchloramphenicol 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 x 10^7/ml in ice-cold PBS with 20 µg of linearized plasmid DNA, and pulsed with 960 µF at 300 V. The cells were harvested and replated in nonselective medium. G418 selective agent (500 µg/ml) was added after 48 hr.

**Gel-shift assays**

Nuclear extracts were prepared [Shapiro et al. 1988] and incubated with 5^2P-end-labeled, double-stranded oligonucleotides: LF-B1, CCTTGGTTAATATTCACC; LF-A1, CAGGCCAGTGTTCTCCTCCGATAAC. Unlabeled competitor oligonucleotides were included in some reactions, including an LF-A2-specific oligonucleotide (GATCCTGTTGCTCCTCCGATAAC). Binding reactions included 5-10 µLg of nuclear extract in 7.5% glycerol, 0.5% aprotinin, 2 mM benzamidine, 0.3 µg/ml of leupeptin, 1 µg/ml of antipain, 1 mM PMSF, 2 µg of poly[dIl-Cl], 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 W/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 µg) 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 α1AT mRNA, a 540-nucleotide radiolabeled antisense RNA probe corresponding to the 3' end of mouse α1AT mRNA was used [from H. Hastie, Edinburgh, UK]. Filters were hybridized at 65°C in 50% formamide, 5× SSPE (1× SSPE = 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate at pH 7.4), 1% SDS, 5× Denhardt's solution (1× Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), and 10 µg/ml each of poly[A] and poly[C] (Pharmacia). After incubation overnight, the filters were washed twice in 2× SSC (1× 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× SSC, 0.1% SDS, at 65°C. The LF-B1 probe was a random-primed 3.6-kb SfiI fragment of pRSVB1. The human α-tubulin probe was a random-primed 1.6-kb cDNA fragment from pk1 [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× SSC, 0.1% SDS, and two 30-min washes at 56°C in 0.1× SSC, 0.1% SDS. Filters were exposed to film for 1–3 days.

**Acknowledgments**

We thank our colleagues at the Hutchinson Center for their comments on the manuscript. G.A.B. is a Leukemia Society of America Fellow. These studies were supported by grant GM26449 from the National Institute of General Medical Sciences.

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Extinction of alpha 1-antitrypsin gene expression in somatic cell hybrids: evidence for multiple controls.

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*Genes Dev.* 1992, 6:
Access the most recent version at doi:10.1101/gad.6.2.316