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Extinction of α1-antitrypsin expression in cell hybrids is independent of HNF1α and HNF4 and involves both promoter and internal DNA sequences

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ABSTRACT

In rat hepatoma × fibroblast somatic cell hybrids, extinction of rat α1-antitrypsin (α1AT) gene expression is accompanied by the loss of liver-enriched transcription factors hepatocyte nuclear factor 1 (HNF1α) and hepatocyte nuclear factor 4 (HNF4). Previous analysis showed that forced expression of functional HNF1α failed to prevent extinction of the rat α1AT locus in cell hybrids. Here I show that ectopic co-expression of HNF1α plus HNF4 fails to prevent extinction of either rat or human α1AT genes in cell hybrids. A 40 kb human α1AT minilocus integrated into the rat genome is fully silenced in cell hybrids in the presence of transacting factors. The integrated α1AT promoter, but not a viral or ubiquitously active promoter, is repressed 35-fold in the cell hybrids. In addition, position effects also contributed to extinction of many integrated transgenes in a cell type-dependent manner. Finally, internal DNA sequences within the human α1AT gene contributed dramatically to the extinction phenotype, resulting in a further 10- to 30-fold reduction in α1AT gene expression in cell hybrids. Thus, multiple mechanisms contribute to silencing of tissue-specific gene expression of the α1AT gene in cell hybrids.

INTRODUCTION

Developmental regulation of tissue-specific gene expression in mammals is a complex process requiring the contribution of both positive and negative regulatory mechanisms. Although our knowledge of positive regulatory controls has advanced greatly in recent years with the discovery of many tissue-specific and tissue-enriched positive *trans*-acting factors, mechanisms responsible for tissue-specific gene repression have largely remained elusive.

In hepatocytes, several liver-enriched transcription factors have been identified that are grouped into four major families, including hepatocyte nuclear factors HNF1, HNF3 and HNF4 and the CCAAT enhancer binding protein (C/EBP) (1). Because binding sites for several of these factors are present within the promoter sequences of a number of liver-specific genes, it is thought that these factors act cooperatively to dictate hepatic gene expression (2). In both dedifferentiated cells derived from hepatoma cells and in hepatoma × fibroblast somatic cell hybrids, the loss of HNF4 and HNF1α expression is commonly observed, while expression of HNF3 and C/EBP family factors remain largely unaffected (3–6). Thus, it has been suggested that the loss of HNF4 and HNF1α is responsible for the general absence of hepatic gene expression in these cell types (6).

Extinction is a term used to describe the dramatic loss of cell-specific gene expression when mammalian cells of distinct lineage are fused to form somatic cell hybrids (7). Although the process of extinction has been observed in many cell hybrid systems, the mechanisms involved in extinction are not understood. Extinction of a number of tissue-specific genes is accompanied by the loss of positive tissue-specific *trans*-acting factors (3,8–13). This apparent correlation between the loss of *trans*-acting factors and the extinction of target genes has been taken to suggest that extinction of tissue-specific genes is simply due to loss of positive-acting factors (14). However, these observations have been largely correlative. Two notable exceptions have been described. First, ectopic expression of HNF4 is sufficient to prevent HNF1α silencing in hepatoma × fibroblast hybrids (4). Second, forced Oct-2 expression prevented extinction of certain B cell-specific genes in B × T cell hybrids (15), two cell types of relatively close developmental lineage. However, Oct-2 expression has not been shown to rescue B cell-specific gene expression in B cell × fibroblast hybrids, suggesting that extinction in hybrids between more distantly related cell types might involve additional regulatory mechanisms.

We have focused on regulation of the liver-specific α1AT gene as a model to understand mechanisms of extinction. This gene serves as an ideal model since: (i) the proximal promoter has been well characterized and shown to require binding by liver-enriched *trans*-acting factors HNF1α and HNF4 (16,17); (ii) α1AT gene mRNA is reduced at least 1000-fold in hepatoma × fibroblast hybrids (13); (iii) the genes encoding HNF1α and HNF4, both extinguished in cell hybrids, have been cloned and characterized (18,19); (iv) the proximal promoter is sufficient to obtain tissue-specific expression of the α1AT gene in transgenic mice (20).

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Our previous studies tested a lack-of-activation model of extinction and involved introduction of cloned HNF1α into rat hepatoma × rat fibroblast cell hybrids with subsequent monitoring of chromosomal α1AT gene expression. These results showed that, while a transiently introduced α1AT promoter could be activated, the endogenous rat α1AT gene remained silent (13). However, these studies assumed that: (i) regulation of the uncharacterized rat α1AT locus was similar to the human α1AT locus; (ii) HNF4, which was shown to be essential for transient activation of the human α1AT promoter in human hepatoma cells (16), is not required for basal activation in rat cell hybrids. In this paper, the well-characterized human α1AT locus is examined to determine the influence of combined HNF1α and HNF4 expression on extinction of human α1AT gene expression in hepatoma × fibroblast hybrids. Results show that HNF4/HNF1α co-expression fails to prevent extinction of the α1AT loci (human or rat) in cell hybrids. Expression of a 40 kb α1AT 'minilocus' is likewise extinguished in similar hybrids. The stable integration of tissue-specific and non-tissue-specific expression cassettes into rat chromatin followed by generation of cell hybrids suggests that extinction of α1AT gene expression involves both the α1AT promoter and internal gene sequences.

MATERIALS AND METHODS

Cell lines and culture conditions

Rat hepatoma cell line FTO2B is a ouabain-resistant, thymidine kinase negative (Oua^r, TK^-) derivative of H4IIEC3 (21). RAT1 is a SV40-transformed rat embryo fibroblast cell line (22). Rn16 (also called RnH1:4-16) is a RAT1 transfectant stably expressing cloned HNF4 (4). RnB1 are pooled RAT1 cells stably expressing HNF1α (13). F(14n)-2 is a cell line derived from FAO-1 hepatoma cells and contains a *neo*-marked human chromosome 14 introduced by microcell-mediated fusion (23). Somatic cell hybrids were generated by PEG-mediated fusion (24) and selection in 500 μg/ml G418 (or HAT) and 3 mM ouabain. All cells were maintained in 1:1 Ham's F12/Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) (Gibco BRL). Neomycin-resistant clones were maintained in 250 μg/ml G418. All cell lines were free of mycoplasma as judged by staining with Hoechst 33258 (25).

Plasmid constructs

Plasmid pATc.1 contains 40 kb of genomic sequences including the human α1AT locus (26). Plasmid pKOneo contains the prokaryotic neomycin phosphotransferase gene (*neo*) under the control of the SV40 late promoter and was provided by K. Yamomota (University of California, San Francisco). Plasmid pPGKβgeo contains the phosphoglyerokinase promoter (27) linked to the β-*geo* gene (a *neo*–β-galactosidase fusion gene; 28) and was provided by P. Soriano (Fred Hutchinson Cancer Research Center, Seattle). Plasmids pCMVβgeo and pAT.36βgeo were generated by replacing the PGK promoter (*Pst*I partial, *Hin*dIII) of pPGKβgeo with a 1157 bp *Pst*I–*Hin*dIII fragment containing the CMV immediate early promoter (from plasmid pCMVlac; 29) or a 366 bp *Bgl*II–*Hin*dIII fragment containing the human α1AT promoter (–366 to –2), respectively. Insertion of the α1AT promoter fragment required blunting the vector and inserts with T4 DNA polymerase prior to ligation. Plasmid pAT7.1 was generated by cloning the 6.8BglII α1AT (about –7100 to –360 bp) sequences into the *Bgl*II site of pAT.36βgeo. pAT5.0βgeo was generated by cloning the α1AT *Hin*dIII fragment (–5400 to –2 bp) from pAT7.1βgeo into pβgeo. pAT1.96 βgeo was generated by cloning a blunted 1.6 kb *Bgl*III–*Eco*RI fragment (–1957 to –366 bp) into the blunted *Bgl*II site (–366 bp) of pAT.36βgeo.

Plasmid pPGK15.3 was generated by partially digesting a 2.85 *Bgl*II fragment (–366 to +2616 bp) of genomic α1AT sequences with *Apa*I, followed by blunting and cloning of the –2 to +2616 bp fragment into vector pGEM2 (Promega). A 12.4 kb *Bgl*II α1AT restriction fragment (+2616 to about +15 000 bp) from pATc.1 (26) was then inserted at the *Bgl*II site to generate the promoterless pd15.3, containing 15.3 kb of genomic α1AT sequences. Plasmid PGK15.3 was made by inserting a 500 bp PGK promoter fragment (*Hin*dIII–*Sma*I) from pPGKβgeo immediately upstream of exon I in the blunted *Sal*I site in the pGEM2 polylinker.

DNA transfections

For stable transfections, both electroporation and lipofection techniques were used. Electroporation was carried out by harvesting exponentially growing cells, resuspending them to 1.2×10^7 cells/ml ice-cold PBS and mixing with 30 μg *Nde*I-linearized plasmid DNA. The cells were electroporated (30) at 960 μF, 300 V using a Bio-Rad Gene Pulser. The cells were incubated in non-selective medium for 48 h and then selective medium was added. After 3 weeks, clones were pooled or picked individually and expanded. For lipofection, liposome-mediated DNA uptake was used according to the manufacturer's instructions (Gibco BRL). After a 5 h incubation in the presence of the DNA/liposome mixture, the medium was replaced with complete medium plus 10% FBS. After 36–48 h, the cells were split 1:20–1:100 into selective medium containing 500 μg/ml (active concentration) G418. Approximately 3 weeks later, transfectants were counted and picked individually or pooled.

Histochemical staining of cell clones for β-gal expression was carried out according to Macgregor *et al.* (31). Cells were fixed in 1% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min, then washed twice with PBS and incubated overnight at 37°C in 5-bromo-4-chloro-3-indolyl-1-β-D-galactopyranoside (X-gal), 4 mM potassium ferro- and ferricyanide, 2 mM $MgCl_2$ and 0.4 mg/ml dimethyl sulfoxide in PBS. Individual clones were scored for the presence of blue precipitate by examination at 100× magnification by light microscopy using a Zeiss telaval 31 microscope.

RNA analysis

RNA was extracted from nearly confluent monolayers in 100 mm dishes by washing the cells twice with saline, harvesting the cells by scraping and pelleting in a microfuge at 12 000 *g* for 10 s. Cells were lysed in NP-40 and extracted twice with phenol/chloroform as described (13). RNA (5 μg) was denatured in 50% formamide at 65°C for 5 min and loaded onto 1% agarose–2.2 M formaldehyde gels. Gels were run at 7 V/cm for 4 h and RNA was transferred to nylon membranes (Zetabind; Cuno Inc.) overnight. The blots were placed in hybridization solution (50% formamide, 5× SSPE; 1× SSPE = 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.4), 1% SDS, 5× Denhardt's solution (1× Denhardt's = 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA) and 10 μg/ml each poly(A) and poly(C) (Pharmacia) for at least 30 min. Probe was added in the same hybridization

solution and the filters were incubated overnight at 42°C. The filters were washed twice for 5 min each in 2× SSC, 0.1% SDS at room temperature, then 30 min in 0.2× SSC, 0.1% SDS at 52°C and exposed to film for 1–5 days. For the murine α1AT riboprobe, filters were prehybridized and probed at 65°C, followed by washing at 65°C in 2× SSC, 0.1% SDS, then at 65°C for 30 min in 0.1× SSC, 0.1% SDS and exposed to film for 1–3 days. Cloned DNA sequences from α-tubulin (Kα-1; 32), HNF1α (18) and human α1AT (p8a1PP9; 33) were labeled with [^{32}P]dCTP by the random hexamer primer method (34). The mouse α1AT probe is a 500 nt [^{32}P]UTP-labeled riboprobe from linearized pAT500.2 (a gift of K. Krauter, Albert Einstein College of Medicine, New York).

To detect HNF4 expression, a 179 nt riboprobe (35) was used. For detection of HNF1α mRNA, a 254 bp fragment corresponding to exon 5 (nt 1867–2121) of rat HNF1α cDNA was used (4). Total cellular RNA (10 µg) was incubated with 1×10^6 c.p.m. of HNF4 and/or HNF1α riboprobe plus 2×10^4 c.p.m. of 18S RNA riboprobe and incubated overnight at 52°C. The 18S riboprobe was synthesized according to the manufacturer's instructions (Ambion Inc.) to generate a low specific activity/high copy number probe. The mixture was digested with RNases T1 + A and protected fragments were resolved on 8% denaturing polyacrylamide gels. The gels were dried and exposed to film for 1–5 days.

RESULTS

Cloned HNF1α fails to prevent extinction of human or rat α1AT loci

Although little is known about the rat α1AT promoter, the human α1AT promoter has been extensively characterized (16,17,20). Promoter mutant studies using clustered point mutations have shown the human α1AT promoter to require both HNF1α and HNF4 for full activity in hepatoma cells (13,36). Ectopic HNF1α expression in hepatoma × fibroblast hybrids failed to prevent extinction of the rat α1AT loci (4,13). In order to determine whether the failure of cloned HNF1α to restore expression of rat α1AT was due to species differences in promoter regulation between rat and human, cell line F(14n)-2 was used in cell hybrid studies. F(14n)-2 is a rat hepatoma cell line containing a *neo*-marked human chromosome 14, on which resides the human α1AT locus (Fig. 1A). F(14n)-2 cells express high levels of human α1AT mRNA (23; results not shown). Cell hybrids were generated by fusion of F(14n)-2 with HNF1α$^+$ rat fibroblasts and grown under selection as described in Materials and Methods. Northern analysis of pooled cell hybrids was carried out using a mouse α1AT riboprobe, which readily detects both human (Fig. 1B, HepG2 dilution panel) and rat α1AT mRNA. As previously reported (13), the rat HNF1α and α1AT loci are fully extinguished in hepatoma × fibroblast hybrids (FR hybrid). Fusion of HNF1α$^+$ rat fibroblasts (RnB1) with F(14n)-2 cells resulted in extinction of both rat and human α1AT expression (Fig. 1B), despite hepatoma levels of HNF1α mRNA being expressed. The failure to detect human α1AT mRNA was not due to probe specificity, as transcripts were readily detected in the human hepatoma line HepG2 (Fig. 1B). Thus, human and rat α1AT loci behave similarly in this cell hybrid system, both being fully extinguished in the presence of HNF1α.

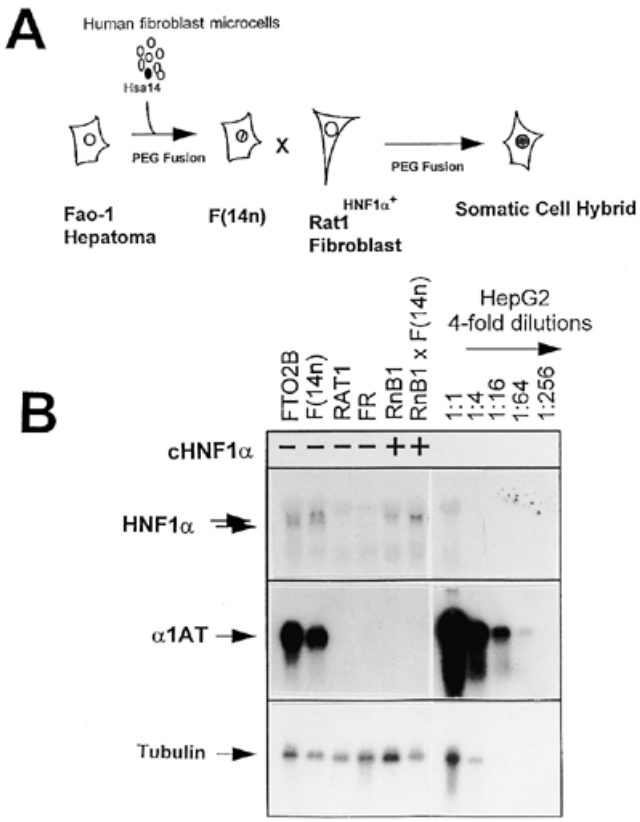

Figure 1. Cloned HNF1α fails to prevent extinction of the rat or human α1AT locus. (**A**) F(14n) cells are Fao-1 hepatoma cells containing a *neo*-marked human chromosome 14 (23). Clone F(14n)-2 cells (containing a complete human chromosome 14) were fused to rat fibroblasts expressing HNF1α and resultant HAT^r/Oua^r hybrids pooled. (**B**) Northern analysis of F(14n)-2 cells fused with HNF1α$^+$ RAT1 fibroblasts (RnB1). An aliquot of 5 µg of total cytoplasmic RNA was size-fractionated, transferred to a nylon membrane and probed successively with HNF1α, α1AT and α-tubulin probes. Two HNF1α gene transcripts, 3.6 and 3.4 kb, are observed in hepatoma cells, whereas only a 3.6 kb transcript is produced from the HNF1α expression plasmid (13). Serial dilutions of human hepatoma HepG2 cell mRNA were included to demonstrate that the mouse α1AT probe cross-hybridizes with the human α1AT RNA. The rat HNF1α probe failed to hybridize to the human HNF1α transcripts. PEG, polyethylene glycol; FTO2B, rat hepatoma cells; RAT1, fibroblasts; FR, FTO2B × RAT1 hybrids; Rnb1, pooled cHNF1-transfected RAT1 fibroblasts.

Combined HNF1α and HNF4 expression fails to prevent extinction of the rat α1AT locus or a human α1AT minilocus

To determine whether the combined effect of HNF1α and HNF4 could prevent extinction of the human α1AT locus, a 40 kb α1AT 'minilocus' plasmid containing the entire human α1AT structural gene plus 8 kb of 5′ and 20 kb of 3′ sequences in a *neo* vector (Fig. 2A) was introduced into rat hepatoma cells and subsequently fused to Rn16 cells. Previous results have shown that fusion of Rn16 (RAT1 fibroblasts stably expressing cloned HNF4) with hepatoma cells prevents extinction of the HNF1α locus (4). Therefore, Rn16 × FTO2B hepatoma hybrids express both HNF4 and HNF1α, although they fail to express the rat α1AT gene (4; Fig. 2B and C). Northern analysis of mRNA from the G418^r pooled hepatoma transfectants showed readily detectable human-specific

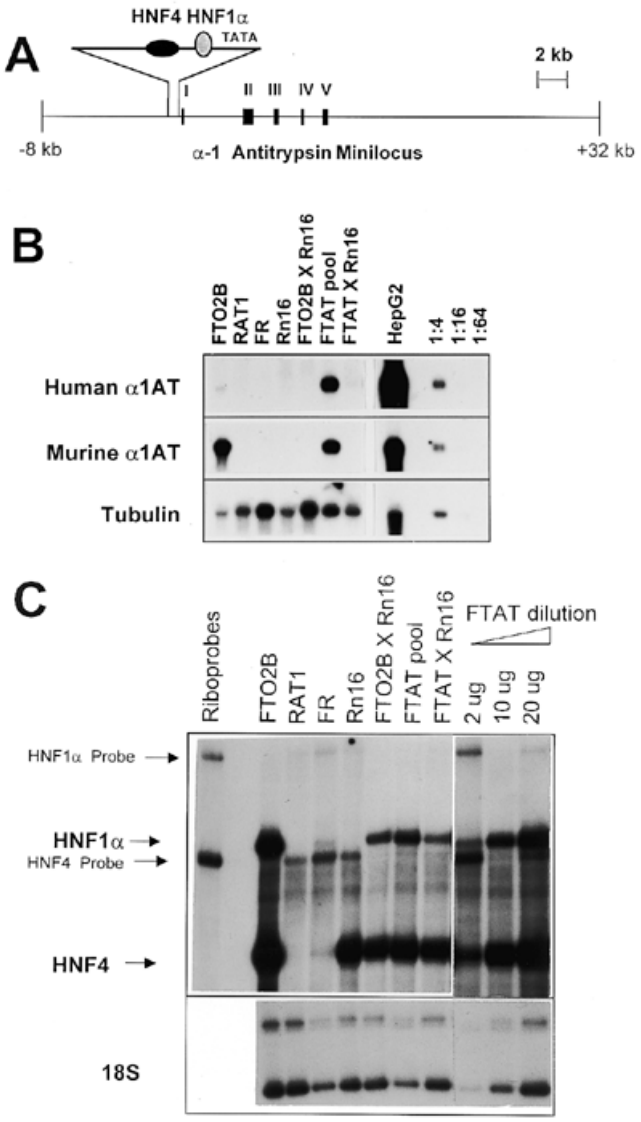

Figure 2. Combined HNF1α and HNF4 expression fails to prevent extinction of rat or human minilocus. (**A**) The α1AT 40 kb 'minilocus' used in this study. Plasmid construct pATc.1 (26), including the 12 kb α1AT gene plus 8 kb of 5′ and 20 kb of 3′ sequences in a *neo*-based plasmid, is shown. Exons I–V are shown with filled boxes. (**B**) Northern analysis of hepatoma × fibroblast cell hybrids. pATc.1 was stably introduced into FTO2B cells and G418^r clones pooled (designated FTAT). FTAT and FTO2B cells were fused with Rn16 cells and G418^r/Oua^r clones pooled. An aliquot of 5 µg of total cytoplasmic RNA was size-fractionated, transferred to a nylon membrane and probed successively with α1AT (murine and human) and α-tubulin probes. The human probe is specific for human α1AT, whereas the murine riboprobe detects both rat and human transcripts. Serial dilutions of human hepatoma HepG2 cell mRNA were included. Rn16 cells are RAT1 cells that constitutively express HNF4. Fusion of Rn16 with hepatoma cells prevents loss of hepatoma HNF1α expression (4). (**C**) RNase protection analysis of HNF4 and HNF1α expression in transfected parental and hybrid cells. An aliquot of 20 µg of each mRNA was hybridized to riboprobes specific for HNF4, HNF1α and 18S rRNA in a single reaction vial. RNase digested fragments were resolved on 8% urea–polyacrylamide gels which were then dried and exposed to film for 1 week (HNF1α and HNF4) or 1 day (18S). FTAT mRNA dilutions were included to verify that band intensity corresponded to levels of mRNA loaded. The undigested HNF4 riboprobe (225 nt) migrates ahead of the HNF1α protected band (251 nt). FTO2B, rat hepatoma cells; RAT1, fibroblasts; FR, FTO2B × RAT1 hybrids; Rn16, cHNF4$^+$ RAT1 fibroblasts; FTAT, FTO2B expressing the human α1AT transgene.

α1AT mRNA (Fig. 2B, FTAT). Fusion of the pooled hepatoma transfectants expressing the human α1AT minilocus with Rn16 cells resulted in hybrids in which the α1AT minilocus was fully extinguished (Fig. 2B). HNF4 and HNF1α genes were both expressed in the hybrids (Fig. 2C). Therefore, co-expression of the two major transactivators of the human α1AT gene, HNF1α and HNF4, fails to prevent extinction of human or rat α1AT gene expression.

Position effects contribute to loss of gene expression in cell hybrids

The observation that forced expression of HNF4 and HNF1α fails to drive expression of the endogenous α1AT gene suggests the possibility that extinction acts through genomic targets in the context of chromatin. In order to test this hypothesis, the influence of position effects on transgene expression in cell hybrids was first determined. To do so, a series of plasmids were constructed containing a series of promoters driving expression of a β-galactosidase–*neo* fusion gene (β-*geo*; 28). Because the β-*geo* gene encodes a single protein encoding both β-galactosidase (β-gal) and G418 resistance functions, it allows for the generation of clones in which 100% of G418^r clones are β-gal positive. These constructs were introduced into both FTO2B hepatoma cells and RAT1 fibroblasts and selected on *neo* expression using G418. Pooled transfectants were then fused to non-transfected partners and the resultant hybrids were assayed for β-gal activity both by histochemical staining of individual clones and by quantitation of enzyme activity in cell extracts. By monitoring both individual clones and overall β-gal expression, a large number of hybrids could be assessed to determine the frequency of transgene extinction due to position effects.

Table 1. Summary of β-*geo* expression in hybrids

Cell line	Description	% Blue[1]	
Parentals			
FTO2B	FTO2B hepatoma	0	(0/66)
RAT1	RAT1 fibroblast	0	(0/74)
Transfectants			
FTPgeo	FTO2B $^{PGK\beta geo}$	100	(22/22)
RPgeo	RAT1$^{PGK\beta geo}$	79	(50/63)
FTCgeo	FTO2B $^{CMV\beta geo}$	100	(23/23)
RCgeo	RAT1$^{CMV\beta geo}$	80	(41/51)
FATBgeo	FTO2B $^{\alpha AT\beta geo}$	100	(58/58)
Cell hybrids			
FTPR	FTO2B $^{PGK\beta geo}$ x RAT1	57	(27/47)
RPFT	FTO2B x RAT1$^{PGK\beta geo}$	77	(57/74)
FTCR	FTO2B $^{CMV\beta geo}$ x RAT1	34	(27/80)
RCFT	FTO2B x RAT1$^{CMV\beta geo}$	78	(70/89)
FAR5	FTO2B $^{\alpha 1AT\beta geo}$ x RAT1	5	(3/60)

[1]Clones with ≥5 blue cells were scored as positive. Clones were scored by light microscopy using a 400× objective. Numbers in parentheses are number of clones scored β-gal positive over total number of clones examined.

Results of β-gal cell staining are shown in Table 1. As expected, the non-transfected parental cell clones grown in non-selective medium scored negative for β-gal expression. Surprisingly, although hepatoma cells transfected with PGK, α1AT or CMV

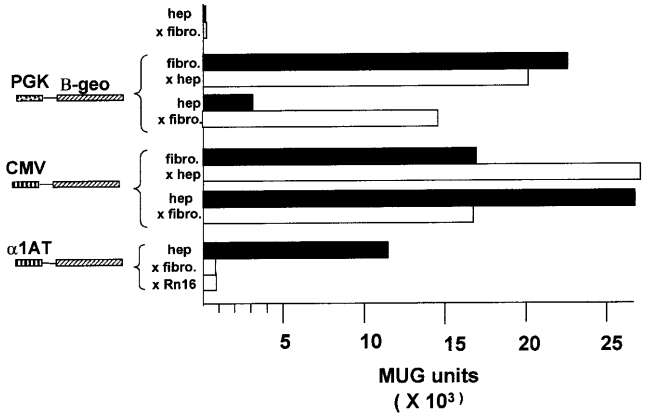

Figure 3. Expression of PGK, CMV and α1AT promoter constructs in hepatomas, fibroblasts and hybrids. Plasmids pPGKβgeo, pCMVβgeo and pAT.36βgeo were transfected into FTO2B and RAT1 cells by electroporation and G418^r clones pooled (normally 30–300 clones/pool). Transfectants were subsequently fused with FTO2B or RAT1 cells and hybrids selected for HAT^r/Oua^r. Cells were harvested and β-gal activity measured by MUG hydrolysis. The filled bars show β-gal activity in parental cell transfectants. Open bars show β-gal levels in subsequent cell hybrids. Hep, FTO2B hepatoma cells; fibro., RAT1 fibroblasts. The values shown are raw values with MUG alone subtracted out. PGK, phosphoglycerolkinase-1 promoter; CMV, cytomegalovirus immediate early promoter.

promoter constructs were 100% β-gal positive, the transfected RAT1 cells were only 79 and 80% β-gal positive for PGK and CMV promoter plasmids, respectively. (The α1AT promoter construct, due to its tissue specificity, was not introduced into the RAT1 cells.) The failure of 20% of the RAT1 transfectants to be scored as β-gal positive is likely due to either the RAT1 cells requiring less *geo* expression to achieve G418 resistance or the apparent cytoplasmic volume of the RAT1 cells being much greater than that of FTO2B, thereby effectively diluting the blue staining observed. The latter explanation appears more plausible, since β-gal activity in lysates from pooled RAT1 transfectants is similar to that from pooled hepatoma transfectants (Fig. 3).

Cell hybrids generated by fusing RAT1$^{CMV\beta geo}$ or RAT1$^{PGK\beta geo}$ cells with FTO2B hepatoma cells were 78 and 77% β-gal positive, respectively. Since the percent β-gal positive clones was nearly identical to the percent β-gal positive RAT1 transfectants prior to fusion, these results suggest that position effects did not account for significant loss of β-gal expression. In contrast, a substantial decrease in β-gal positive clones was observed when FTO2B$^{CMV\beta geo}$ and FTO2B$^{PGK\beta geo}$ transfectants were fused to RAT1 cells, resulting in 34 and 53% β-gal positive hybrids, respectively (compared with 100% β-gal positive in the original transfectants). Thus, these results suggest that a number of extinction phenotypes (loss of β-gal expression) in the stable transfectants are due to position effects in the hepatoma genome. However, silencing due to position effects was infrequently observed when transgenes were integrated into the RAT1 fibroblasts.

Quantitation of β-gal activity in cell lysates showed that PGKβgal and CMVβgal transgene expression, although variable, were highly active in both the fibroblast and hepatoma transfectants (Fig. 3). The subsequent hybrid cells continued to express the β-gal gene at levels within 3-fold of the β-gal-expressing parent. Thus, an extinction phenotype was not observed in pooled hybrids with either CMV or PGK promoter transgenes.

The integrated α1AT promoter is poorly expressed in hybrids in the presence of HNF4 and HNF1α

In contrast to the above results using PGK and CMV promoters, fusion of FTO2B$^{\alpha 1AT\beta geo}$ cells with RAT1 cells resulted in only 5% β-gal positive hybrids, consistent with the lack of α1AT promoter activity in cells lacking HNF4 and HNF1α (13). Those clones which were scored as β-gal positive may have been hybrids segregating fibroblast chromosomes. Indeed, cell morphology suggests this to be the case (results not shown). β-gal activity in cell lysates of FTO2B$^{\alpha 1AT\beta geo}$ × RAT1 or FTO2B$^{\alpha 1AT\beta geo}$ × Rn16 showed β-gal levels slightly over background, suggesting that the presence of HNF4 and HNF1α fails to be sufficient for optimal α1AT promoter activity.

To examine α1AT promoter activity in individual hybrid clones, a series of α1AT promoter deletion constructs driving the β-gal gene were stably integrated into the FTO2B hepatoma cells and individual clones subsequently fused to the Rn16 cells. β-gal expression was then monitored in cell lysates in parental cells and hybrids. Although β-gal expression was highly variable, plasmids containing longer α1AT promoter sequences (pAT7.1 and pAT5.0) generally expressed higher levels than those with shorter sequences (pAT1.96 and pAT.36) (Fig. 4). In all cases, fusion with Rn16 cells resulted in hybrids expressing very low levels of β-gal, averaging a 33- to 38-fold reduction regardless of the length of α1AT sequence used. Most clones were found to contain a single copy of the α1AT transgene although a number of clones containing the 7.1 promoter construct have multiple copies (results not shown). Lack of promoter activity was not due to loss of HNF4 or HNF1α expression, since hybrid cells express HNF4 and HNF1α mRNA (as determined by RNase protection analysis of representative samples) (Fig. 5).

Internal α1AT DNA sequences contribute to α1AT extinction in cell hybrids

The above results suggest that additional constraints beyond reduced promoter activity are responsible for the complete lack of α1AT mRNA in hepatoma × fibroblast hybrids. To determine whether internal α1AT DNA sequences contribute to the extinction phenotype, the PGK promoter was fused to a 15.3 kb region of the human α1AT locus (Fig. 6A). This construct contains the PGK promoter at the transcription start site of the α1AT genomic sequences (at position –2). This plasmid was co-transfected with a *neo* plasmid into FTO2B cells and G418^r clones either pooled or picked individually. Approximately half of the clones expressed human α1AT mRNA at levels much higher than the endogenous gene (Fig. 6B). The pooled clones as well as two high expressing clones were then fused to RAT1 fibroblasts. The resultant hybrid cells showed a marked reduction in α1AT mRNA levels compared with the parental levels (Fig. 6C), ranging from an 11- to 30-fold reduction in expression (as determined by PhosphorImaging). Southern analysis verified that loss of the transgene did not occur (results not shown). Thus, the internal α1AT DNA sequences appear to contribute substantially to the α1AT extinction phenotype in cell hybrids. Whether this effect is transcriptional or post-transcriptional has yet to be investigated.

DISCUSSION

The silencing of tissue-specific gene expression in mammalian cell hybrids is thought to reflect mechanisms responsible for

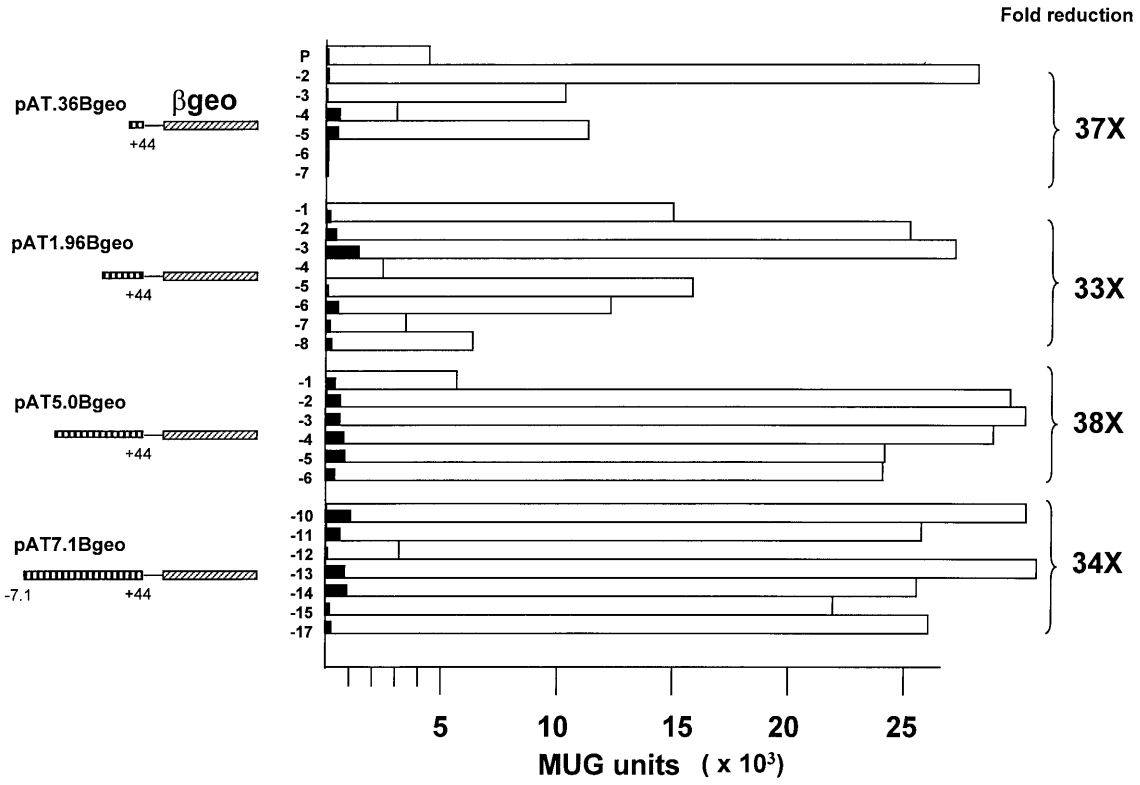

Figure 4. Expression of α1AT promoter deletion constructs in hepatoma transfectants and cell hybrids. FTO2B cells were transfected via lipofection with each plasmid shown. G418^r clones were picked individually. Transfectants were subsequently fused to Rn16 cells and HAT^r/Oua^r hybrids pooled. Cells were harvested and β-gal activity measured by MUG hydrolysis. The open bars show β-gal activity in transfected hepatoma cells. Filled bars show β-gal values in subsequent hybrids. Numbers to the right show average reduction in promoter activity measured in each set of transfectants, ignoring those with MUG values <2-fold above parental FTO2B values. P, pooled (>40 clones) pAT.36Bgeo transfectants.

limiting inappropriate gene activity in mammalian cells during differentiation and/or development (7). The extinction phenotype in hepatoma × fibroblast hybrids is complete, global (affecting all or nearly all tissue-specific genes) and reversible (upon loss of chromosomes from the non-expressing parental cell) (7,37). Evidence from these and other experimental systems suggest that the mechanisms responsible for this gene silencing in cell hybrids may involve lack-of-activation (4,15,38), dominant supression (39,40) or a combination of both.

The human α1AT promoter has been extensively studied in the context of tissue specificity by introduction of constructs (13,16,17,20,41) and in the mouse germline (20,26,42). Notably, the minimal promoter appears to contain the regulatory signals sufficient for tissue-specific expression (20). The minimal promoter contains functional binding sites for liver-enriched factors HNF1α and HNF4. The loss of both of these factors in hepatoma × fibroblast hybrids suggested the possibility that α1AT gene silencing could be explained by a loss-of-activation phenotype. Results presented here argue against this simple model, showing that constitutive HNF4 and HNF1α expression is not sufficient to maintain expression of the human or rat α1AT genes. The observed 35-fold decrease in promoter activity in cell hybrids, while dramatic, does not account for the complete extinction phenotype of >1000-fold reduction in α1AT mRNA levels (13). Indeed, the majority of hybrid clones analyzed continued to express the α1AT–β-*geo* transgenes, albeit at low levels. In a number of cell hybrids, promoter activity was undetectable, although these cases are likely due to position effects also observed with two ubiquitously active promoters (the cellular PGK promoter and cytomegalovirus immediate early promoter) being silenced in >40% of the cell hybrids (discussed below).

HNF4 and HNF1α are systematically absent in dedifferentiated rat hepatoma cells and in hepatoma × fibroblast somatic cell hybrids (5,6,13,43), both of which lack the hepatic phenotype. However, expression of other liver factors (HNF3 and C/EBP) remains largely unchanged in these cell systems (5,6,44). This suggests that HNF4 and HNF1α are required for the hepatic phenotype. The reported ability of HNF4 expression to activate hepatic gene expression in certain hepatoma variant cells (44,45) supports this hypothesis. However, these factors are not necessarily sufficient for hepatic gene expression. Ectopic expression of HNF4 and/or HNF1α fails to restore hepatic gene expression in certain hepatoma variants (35) and in cell hybrids (4; this manuscript). In addition, hepatoma variant lines have been described in which hepatic gene expression is absent despite normal expression of endogenous HNF4 and HNF1α genes (46). Thus, additional regulatory constraints, either positive or negative, appear to dictate the ability of hepatic genes to be expressed. The α1AT promoter studies presented here failed to identify DNA sequences within the α1AT promoter that are targeted during extinction. However, these studies cannot rule out a factor that acts to prevent gene activation in the context of chromatin.

Silencing of transgenes due to position effects dependent upon site of integration is well known (47,48), although the mechanisms

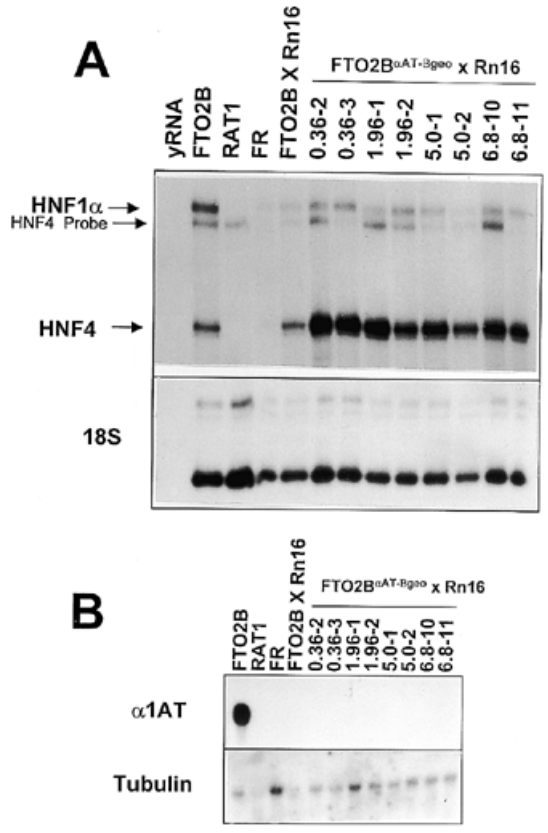

Figure 5. (**A**) HNF4 and HNF1α expression in FTO2B$^{\alpha 1AT\beta geo}$ × Rn16 hybrids. RNA from representative cell hybrids shown in Figure 4 were assayed for HNF1α and HNF4. An aliquot of 10 µg of each mRNA was hybridized to riboprobes specific for HNF4, HNF1α and 18S rRNA in a single reaction vial. RNase digested fragments were resolved on 8% urea–polyacrylamide gels which were then dried and exposed to film for 1 week (HNF1α and HNF4) or 1 day (18S). The location of undigested probes and RNase protected fragments are shown. yRNA, yeast RNA as a non-specific control. (**B**) Northern analysis of mRNA from FTO2B$^{\alpha 1AT\beta geo}$ cells. An aliquot of 5 µg of total cytoplasmic RNA was size-fractionated, transferred to a nylon membrane and probed successively with murine α1AT and α-tubulin probes. FTO2B, rat hepatoma cells; RAT1, fibroblasts; FR, FTO2B × RAT1 hybrids; Rn16, cHNF4^{+} RAT1 fibroblasts.

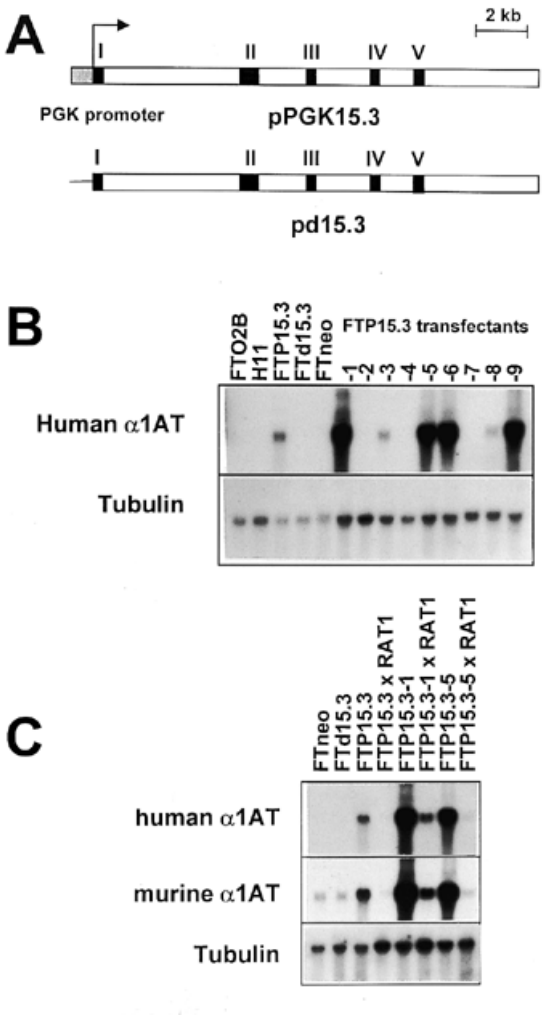

Figure 6. Extinction of a PGK–α1AT construct in cell hybrids. (**A**) Plasmid pd15.3 contains a promoterless 15.3 kb α1AT minilocus. pPGK15.3 contains the PGK promoter fused to α1AT genomic sequences at position –2 of intron I. Positions of exons I–V are shown with closed bars. (**B**) FTO2B cells were co-transfected with pPGK15.3 (or pd15.3) plus pKOneo and G418^{r} clones either picked individually or pooled. An aliquot of 5 µg of total cytoplasmic RNA was size-fractionated, transferred to a nylon membrane and probed successively with human α1AT and α-tubulin probes. FTO2B, rat hepatoma cells; H11, α1AT^{-} hepatoma variant cells (35). (**C**) Select FTO2B transfectants expressing human α1AT were fused with RAT1 fibroblasts and HAT^{r}/Oua^{r} hybrids selected, pooled and expanded. Cytoplasmic RNA was size-fractionated, transferred to a nylon membrane and probed with human and murine α1AT and tubulin (as an RNA loading control) probes.

responsible are not fully understood. Position effects were examined in the context of gene extinction in this study. Results suggest that loss of transgene expression is greatly influenced by the parental cell type into which the transgene is introduced prior to fusion. β-gal expression cassettes containing CMV and PGK promoters integrated into the fibroblast chromosomes were rarely silenced when fused to hepatoma cells. In contrast, integration of trangenes into hepatoma chromatin followed by fusion with fibroblasts resulted in loss of transgene expression in >40% of the hybrid cells. The extent of this loss of transgene expression was not determined. However, based upon intensity of blue staining in the hepatoma transfectants compared with subsequent hybrids, the effect is substantial. The reason for these differences in observed position effects between fibroblast and hepatoma integrants is not known. It is possible that this is due to the fact that the hepatoma cells express a large number of liver-specific genes, the majority of which are extinguished in cell hybrids. If transgene integration occurs preferentially into highly active chromatin, then these insertions may be down-regulated because the chromatin changes in liver-specific genes during the process of gene extinction. This hypothesis has not been formally tested, but it would be useful to know whether genes are silenced due to proximity with regions of chromatin that are extinguished.

Each of the α1AT promoter deletion mutants tested (–7100 to –360 bp) behaved in a similar fashion in cell hybrids, in that promoter activity was reduced by an average of 35-fold with each deletion plasmid. Thus, sequences responsible for negative regulation of the α1AT promoter in cell hybrids were not identified. It is still possible that a factor(s) in the cell hybrids interferes with the ability of the minimal promoter to function in

the presence of HNF4 and HNF1. Therefore, factors that can modulate chromatin, such as methylases, histone acetylases or 'propagator proteins' (49,50), may play a central role in α1AT gene inactivation.

It was observed that a transgene containing the PGK promoter fused to the α1AT genomic sequences, while highly expressed in hepatoma cells, was strongly repressed in cell hybrids. This is in contrast to the PGK–β-*geo* transgene, which remained active in similar cell hybrids, suggesting that sequences within the α1AT locus are targets for repression in the cell hybrids. The α1AT mRNA expressed by the PGK–α1AT construct is the expected size (1.4 kb), indicating that transcriptional initiation and splicing occurred normally. This result is significant, because it suggests that extinction phenotypes that have been reported in cell hybrids may be due to sequences within the gene, acting at either transcriptional or post-transcription levels, rather than through promoter sequences. Several examples of internal transcriptional regulatory elements have been documented in mammalian genes, including intronic and dominantly acting regulatory sequences involved in extinction of immunoglobulin gene expression in B cell × fibroblast hybrids (39).

The extinction phenotype presents an all-or-none pattern, reminiscent of position effect variegation in *Drosophila* (in which cells are locked into one of two states) and in development-specific expression of the human β-globin gene cluster (49,51). HNF1α and HNF4 (plus other liver-enriched transcription factors) may provide the environment for activation, but further chromatin modulation may be required in order to provide accessibility of these factors to the α1AT gene. Further studies examining the state of chromatin in and around the α1AT locus in different cellular contexts (in the presence and absence of HNF4 and HNF1α) may provide insight into higher order regulation of tissue-specific genes. It is worthwhile to note that the human α1AT gene lies within a serine protease gene cluster containing genes encoding α1-antichymotrypsin, corticosteroid-binding globulin and protein C inhibitor as well as an α1-antitrypsin-related gene (52). Remarkably, all of these genes have nearly identical organization and intron–exon junctions (53,54). These similarities suggest the possibility of common regulatory mechanisms and thus possible shared DNA motifs through which extinction mechanisms may act.

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