

## Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice

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### SUMMARY

Targeted expression of oncogenes in transgenic mice can immortalize specific cell types to serve as valuable cultured model systems. Utilizing promoter regions from a set of genes expressed at specific stages of differentiation in a given cell lineage, we demonstrate that targeted oncogenesis can produce cell lines representing sequential stages of development, in essence allowing both spatial and temporal immortalization. Our strategy was based on our production of a committed but immature pituitary gonadotrope cell line by directing expression of the oncogene SV40 T antigen using a gonadotrope-specific region of the human glycoprotein hormone  $\alpha$ -subunit gene in transgenic mice. These cells synthesize  $\alpha$ -subunit and gonadotropin-releasing hormone (GnRH) receptor, yet are not fully differentiated in that they do not synthesize the  $\beta$ -subunits of luteinizing hormone (LH) or follicle-stimulating hormone (FSH). This observation led to the hypothesis that targeting oncogenesis with promoters that are activated earlier or later in development might immortalize cells that were more primitive or more differentiated, respectively.

To test this hypothesis, we used an LH $\beta$  promoter to immortalize a cell that represents a subsequent stage of gonadotrope differentiation (expression of  $\alpha$ -subunit, GnRH receptor, and LH  $\beta$ -subunit but not FSH  $\beta$ -subunit). Conversely, targeting oncogenesis with a longer fragment of the human  $\alpha$ -subunit gene (which is activated earlier in development) resulted in the immortalization of a progenitor cell that is more primitive, expressing only the  $\alpha$ -subunit gene. Interestingly, this transgene also immortalized cells of the thyrotrope lineage that express both  $\alpha$ - and  $\beta$ -subunits of thyroid-stimulating hormone and the transcription factor GHF-1 (Pit-1). Thus, targeted tumorigenesis immortalizes mammalian cells at specific stages of differentiation and allows the production of a series of cultured cell lines representing sequential stages of differentiation in a given cell lineage.

Key words: transgenic mice, targeted oncogenesis, gonadotropins, thyroid-stimulating hormone, pituitary, mouse

### INTRODUCTION

Organogenesis in mammals is a highly complex process that has been difficult to study experimentally. The anterior pituitary gland is an excellent model system for the study of cellular differentiation during organogenesis since the appearance of its five constituent endocrine cell types occurs in a well-defined order based on the expression of specific hormone genes. Thus, defining the regulation of hormone gene expression at various stages of anterior pituitary development will also enable us to elucidate the mechanisms of regulation of cytodifferentiation in this multifunctional organ. To this end, we have focused on creating cultured models that reflect distinct steps in anterior pituitary development.

The anterior pituitary is derived from a single epithelial layer referred to as Rathke's pouch (Simmons et al., 1990). The earliest marker of anterior lobe differentiation is the glycoprotein hormone  $\alpha$ -subunit gene which is expressed at approximately embryonic day 11.5 (E11.5) of gestation in the mouse (Japon et al., 1994). Beginning on E13.5, there is a clear progression of cell differentiation initiated by the commitment of the corticotrope lineage marked by the expression of the proopiomelanocortin (POMC) gene. Differentiation of the thyrotrope lineage occurs on E14.5 with the expression of thyroid-stimulating hormone (TSH)  $\beta$ -subunit. The somatotrope and lactotrope cells arise on E15.5, with the expression of growth hormone (GH) and prolactin (Prl) genes, respectively. The last lineage to emerge is the gonadotrope. Its differentiation pathway can be subdivided into two stages marked by the expression of the individual  $\beta$ -subunits of luteinizing hormone (LH) on E16.5 and follicle stimulating hormone (FSH) on E17.5. The individual  $\beta$ -subunits bind noncovalently to the common  $\alpha$ -subunit to form the mature glycoprotein hormones, TSH, LH and FSH, and, appropriately, the expression of the

$\alpha$ -subunit is restricted to the gonadotrope and thyrotrope lineages in adult pituitary.

Directed oncogenesis in transgenic mice allows the production of differentiated immortal cell lines for the study of tumorigenesis, gene expression, and the molecular and cellular biology of specific tissue types (Camper, 1987; Hanahan, 1989). Previously, we demonstrated that a transgene with 1.8 kb of the 5' regulatory region of the human  $\alpha$ -subunit gene linked to the coding region for the oncogene SV40 T antigen immortalized immature gonadotrope cells (the  $\alpha$ T3-1 cell line) that expressed the  $\alpha$ -subunit gene and responded to the gonadotrope-specific hypothalamic-releasing factor, gonadotropin-releasing hormone (GnRH), but did not express the  $\beta$ -subunits of either LH or FSH (Windle et al., 1990). This result suggested that it might be possible to immortalize cells at various stages of differentiation with hybrid oncogenic transgenes utilizing the regulatory regions of genes expressed at distinct stages of development. To test this hypothesis, we have used hybrid genes consisting of either the human or rat LH  $\beta$ -subunit promoter linked to T antigen to immortalize a cell that succeeds the  $\alpha$ T3-1 cell in the gonadotrope lineage. Conversely, to capture a more primitive  $\alpha$ -subunit-expressing cell that represents an earlier stage of anterior pituitary development than the  $\alpha$ T3-1 cell, we introduced the oncogene T antigen under the control of a longer portion (5.5 kb) of the 5' flanking region of the human  $\alpha$ -subunit gene into transgenic mice. Using this approach, we have produced cell lines that express either the  $\alpha$ -subunit alone, TSH ( $\alpha$  and TSH $\beta$ ), or LH ( $\alpha$  and LH $\beta$ ) genes. These cell lines maintain the phenotypic markers of their developmental stages and represent a progression of differentiation within the glycoprotein hormone-producing cell lineages. They provide novel model systems in which the molecular mechanism of pituitary cellular differentiation can be dissected. This study demonstrates that directed tumorigenesis controlled by regulatory regions that specify cell-type and temporal expression can result in the immortalization of cells at distinct stages of differentiation in a given developmental cell lineage.

## MATERIALS AND METHODS

### Plasmid construction

The  $\alpha$ -Tag plasmid is as described (Windle et al., 1990). Digesting a parent Bluescript vector (p5.5 $\alpha$ BSSK+) with *Bam*HI followed by a partial digest with *Eco*RI released the  $\alpha$ -subunit promoter spanning from approximately -5.5 kb to +49 (Fiddes and Goodman, 1981). This fragment was subsequently cloned into the *Eco*RI and *Bam*HI sites upstream of the SV40 early region coding sequence for both large and small T antigens in the identical vector used to generate the 1.8 kb 5'  $\alpha$ -subunit/T antigen plasmid (Windle et al., 1990). The human gene for LH $\beta$  contains only 10 bases between the mRNA start site and the ATG initiation codon. *Sty*I restriction enzyme cleaves this region at +5. *Sty*I partial digest was treated with *E. coli* large fragment polymerase to repair the four base overhang, leaving a blunt end at +9. This was joined to the T antigen coding sequence with a *Xba*I linker at the *Bgl*III site of SV40. Similarly, a 1.8 kb fragment of the 5' flanking region of the rat LH $\beta$  gene was isolated by digestion with *Hind*III and *Xho*I of a pUC19 plasmid (kindly provided by J. L. Roberts) which has an *Xho*I linker inserted at +5 of the rat LH $\beta$  gene. This fragment was cloned with the same SV40 T antigen DNA as described above. The structures of the human  $\alpha$ -subunit/T antigen, and rat and human LH $\beta$ /T antigen transgenes are diagrammed in Fig. 1.

### Transgenic animals

The transgene fragments were isolated with the appropriate restriction enzymes and purified after gel electrophoresis by binding to glass beads (BIO101, Inc., La Jolla, CA: GeneClean). The fragments were quantitated and diluted to a concentration of 2  $\mu$ g/ml in injection buffer consisting of 10 mM Tris, pH 7.4 and 0.25 mM EDTA. The DNA was injected into the pronuclei of fertilized one cell mouse embryos derived from matings of CB6F<sub>1</sub>/J mice (C57Bl/6J  $\times$  BALB/cJ; Jackson Laboratory, Bar Harbor, ME or Harlan Sprague Dawley, Indianapolis, IN). The injected embryos were implanted into CD-1 or ICR pseudopregnant hosts (Hogan et al., 1986). Genomic DNA was isolated from a small portion of the tail and analyzed for the presence of the transgene by Southern blot (Maniatis et al., 1982). For those transgenic lines that could be established from a fertile founder animal, transgene transmission was observed at approximately 50%.

### Derivation of cell lines

Tumors were dissected from the sella turcica and gross autopsies were performed to identify any ectopic tumor formation. A small portion of each pituitary tumor was retained for RNA analysis and the remainder was minced and dispersed in Hanks Balanced Salt solution containing 10 mg/ml collagenase (Cooper Biomedical, Malvern, PA), and 10  $\mu$ g/ml DNase I (Sigma, St Louis, MO) at 37°C for 30 minutes. The cells were washed in PBS and subsequently plated in Dulbecco's Modified Eagles Medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 4.5 mg/ml glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO). The floating and weakly adherent cells were transferred to fresh plates every 2 days until the cells were freed of fibroblast contamination. After approximately 2-6 months, the endocrine cells became more adherent and began to grow on plastic. In the case of the T $\alpha$ T1 line, the cells were seeded on Matrigel-coated plates (Collaborative Biomedical Products, Bedford, MA) which facilitated adhesion. Matrigel was diluted 30-fold prior to coating the plates. Subclones of each cell line were subsequently identified following clonal dilution or colony isolation. The cells are maintained in the above-mentioned medium in an environment of 5% CO<sub>2</sub>.

### Northern analysis

Total RNA was isolated by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) and 10  $\mu$ g of each sample was electrophoresed in a 1% agarose gel containing formaldehyde. Equivalent loading was further verified by ethidium bromide staining (data not shown). Following transfer onto nylon membrane (Genescreen, NEN Research Products, Boston, MA), the RNA was fixed by UV-crosslinking using a Bio-Rad UV chamber (Bio-Rad Laboratories, Richmond, CA). Membranes were hybridized with a <sup>32</sup>P-labeled cDNA probe overnight at 55°C in a 25% formamide solution. DNA probes to the hormones designated in the figure legends were radioactively labeled by random priming (Feinberg and Vogelstein, 1983, 1984). Excess probe was removed by washing at 65°C with 0.2 $\times$  SSPE (36 mM NaCl, 2 mM sodium phosphate, 0.2 mM EDTA) and 0.1% sodium dodecyl sulphate. Blots were stripped by boiling in 1% glycerol, 0.5% sodium dodecyl sulfate and 2 mM EDTA before rehybridization with subsequent probes.

### Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as previously described by Eraly and Mellon (1995), and stored at -80°C. Protein concentrations were determined using Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA). In a total volume of 20  $\mu$ l, 5  $\mu$ g of nuclear extract was mixed with 1  $\mu$ g poly(dI-dC) as nonspecific competitor and 1% Ficoll 400 in a buffer containing 75 mM NaCl, 15 mM Tris, pH 7.4, 1.5 mM EDTA, 4% glycerol, 20  $\mu$ g/ml bovine serum albumin and 1.5 mM DTT. Following preincubation at 4°C for 20 minutes, 1 ng of radio-labeled oligonucleotide was added to each reaction mix and incubated

at room temperature for 20 minutes. Competitions were performed using 100 ng of the indicated unlabeled oligonucleotides in each binding reaction. The oligonucleotides were labeled by filling in overhanging ends with Klenow enzyme in the presence of [ $\alpha$ - $^{32}$ P]ATP. The  $\alpha$ GSE oligonucleotide (Barnhart and Mellon, 1994) corresponds to positions -221/-206 of the human  $\alpha$ -subunit promoter. The mGSE oligonucleotide has the identical sequence as GSE, except that the nucleotide pair CC in positions 6 and 7 of the oligonucleotide are changed to TT. The SF-1 oligonucleotide has the sequence 5'-CTCT-TAGCCTTGAGC-3', and corresponds to positions -50 to -36 of the human cholesterol side-chain cleavage enzyme gene (Rice et al., 1990). The GHF-1 oligonucleotide is a consensus GHF-1-binding site and has the sequence ATTACATGAATATTCAT with *Eco*RI compatible ends (kindly provided by Dr Holly Ingraham). The reaction mixture was electrophoresed in a 4% acrylamide:bis (40:2) gel and visualized by autoradiography.

## RESULTS

### Tissue-specific tumorigenesis in transgenic mice

To test the hypothesis that cells representative of different stages of a given cell lineage could be immortalized by targeting expression of an oncogene using regulatory regions of genes expressed sequentially during development, we generated transgenic mice using various glycoprotein gene 5' regulatory regions linked to the early coding region of SV40. This portion of the virus produces both the large and small T antigens by differential splicing (herein referred to as T antigen). Fig. 1 illustrates the structure of the transgenes that were injected into fertilized eggs. The resultant founder mice and mouse lines are enumerated in Tables 1 through 4. Many of the transgenic mice were infertile or had reduced fertility with age, especially the females. This is likely due to the formation of pituitary tumors that occurs progressively with age.

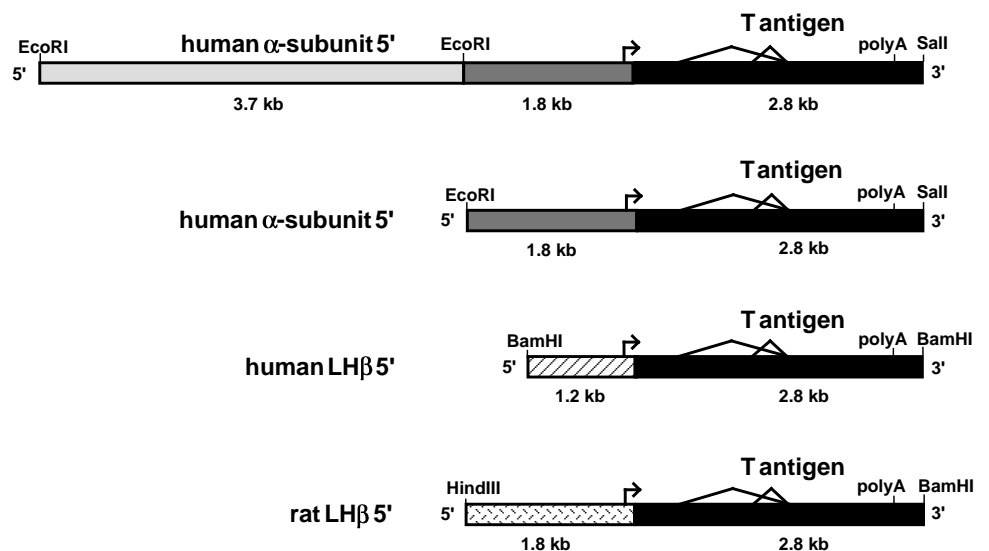
### Hormone gene expression in pituitary tumors

Targeting T antigen expression with 1.8 kb of the 5' flanking region of the human  $\alpha$ -subunit gene resulted in pituitary tumor formation in thirteen and infertility in nine of seventeen lines of transgenic mice (Table 1), termed the  $\alpha$ T lines (Horn et al., 1992). Histological examination of these tumors showed them to possess several cell types not found in normal pituitary glands including a 'giant' cell that expressed the  $\alpha$ -subunit and either the TSH $\beta$  or LH $\beta$  subunit genes (Schechter et al., 1992). The cell lines derived from these tumors, however, expressed only the  $\alpha$ -subunit gene (Windle et al., 1990). Thus, to obtain a more global assessment of the transformed cells within the tumors, we performed northern analysis of total RNA isolated from

tumor fragments from four  $\alpha$ T transgenic lines ( $\alpha$ T-1 was a single animal which did not breed to allow creation of a mouse line). Hybridization with cDNA probes of the seven pituitary hormone markers demonstrated that these tumors expressed varying levels of the endogenous  $\alpha$ -subunit mRNA (Fig. 2). The partner glycoprotein hormone  $\beta$ -subunits of TSH, LH and FSH were not detectable, demonstrating that the tumors were composed predominantly of cells that only expressed  $\alpha$ -subunit but not  $\beta$ -subunit genes. As expected, GH and POMC mRNAs were not detectable with the exception of a weak signal for POMC in the  $\alpha$ T12-2 tumor. This may be due to inclusion of some tissue from the intermediate lobe of the pituitary in the tumor sample. It is likely that some portion of the tumors contained normal pituitary tissue that was unidentifiable and unavoidable upon initial dissection.

Surprisingly, the majority of the tumors did express Prl RNA. This implies that either the tumor cells expressed both  $\alpha$ -subunit and Prl genes, or that normal pituitary cells are intermingled with transformed cells. Since the lactotrope (the cell that expresses Prl) is the only anterior pituitary cell type that is under negative growth regulation by the hypothalamus (through dopamine), high Prl expression may be due to physical disruption of hypothalamic regulation of normal lactotrope cells by the tumor followed by expansion of the lactotrope population due to derepression of mitosis. High levels of Prl mRNA would be due to a lack of hypothalamic dopaminergic inhibition of Prl expression and a lack of inhibition of mitosis (Weiner et al., 1988) in nontransformed lactotropes due to compression of the hypothalamic stalk by the tumor and consequent lack of dopamine. In support of this, the cell lines derived from these tumors only expressed  $\alpha$ -subunit and did not express Prl (Windle et al., 1990).

To generate tumors that contain cells that are more differentiated than the cells in the  $\alpha$ T tumors, i.e. that express the



**Fig. 1.** Structure of the glycoprotein hormone  $\alpha$ -subunit and LH $\beta$  T antigen transgenes. Plasmids are described in Materials and Methods. The transgenes contain promoter regions of the human  $\alpha$ -subunit gene, the human  $\beta$ -subunit gene or the rat  $\beta$ -subunit gene linked to the SV40 early region (Windle et al., 1990). The SV40 fragment contains the protein coding region for large and small T antigens, the translation initiation and transcription termination sites, but lacks the SV40 early promoter. The transgenes were excised from the plasmid vector with the appropriate restriction enzymes for microinjection into fertilized mouse oocytes.

**Table 1. Transgenic mice carrying the 1.8 kb human glycoprotein hormone  $\alpha$ -subunit promoter linked to T antigen\***

Mouse	Pituitary tumors†	Ectopic tumors‡	Reproductive status§
$\alpha$ T-1	93 d		infertile
$\alpha$ T-2	92 d		infertile
$\alpha$ T-3	49 d		sexually immature
$\alpha$ T-4	93 d		infertile
$\alpha$ T-5	none		fertile
$\alpha$ T-6	143 d		one litter of 2 pups
$\alpha$ T6-2	101 d		infertile
$\alpha$ T-7	272 d	thymus	fertile
$\alpha$ T7 line	110-250 d	pancreas, lymph node	M fertile, F infertile
$\alpha$ T-8	none		fertile
$\alpha$ T-9	105 d		fertile; no transmission
$\alpha$ T-10	178 d		infertile
$\alpha$ T-11	98 d		infertile
$\alpha$ T-12	134 d		fertile
$\alpha$ T12 line	~140 d		M fertile, F infertile
$\alpha$ T-13	43 d		N.D.
$\alpha$ T14 line	420 d	pancreas	fertile
$\alpha$ T-15	none		fertile; no transmission
$\alpha$ T-16	none		fertile
$\alpha$ T-17	252 d		infertile

\*Reproduced from (Horn et al., 1992) with permission.

†Animals were killed or died due to pituitary tumors at the age indicated.

‡Tumors, in addition to pituitary tumors, that were identified upon autopsy.

§Indicates the fertility profile of the founder and transgenic lines. (M) male, (F) female.

LH $\beta$ -subunit, 1.2 kb of the human (hLT mice) and 1.8 kb of the rat LH $\beta$  5' flanking DNA (rLT mice) were used to drive T antigen expression in transgenic animals (Tables 2, 3). Eight out of twelve transgenic mouse lines generated using the human promoter developed pituitary tumors or hyperplasia (hLT mice, Table 2). Northern blot analysis of tumor fragments shows that glycoprotein hormone subunit

**Table 2. Transgenic mice carrying the human LH $\beta$  promoter linked to T antigen**

Mouse line	Pituitary tumors*	Ectopic tumors†	Reproductive status‡
hLT1	small tumors ~1.5 yrs		fertile
hLT2	none		fertile
hLT3	hyperplasia 150-180 d	retina§	fertile
hLT4	none		fertile
hLT6	none		fertile
hLT7	none to slight hyperplasia		fertile
hLT9	occasional tumors ~390 d	2 thymus	fertile
hLT11	slight hyperplasia		fertile
hLT12	none		fertile
hLT13	small tumor 450 d	lymph node, liver	fertile
hLT14	small tumors 420-450 d		fertile
hLT15	small tumors ~390 d		fertile

\*Animals were killed or died due to pituitary or other tumors at the age indicated.

†Tumors, in addition to pituitary tumors, that were identified upon autopsy and occur with a much lower penetrance (except for retinoblastoma in hLT3, see below§).

‡Indicates the fertility profile of the founder and transgenic lines. (M) male, (F) female.

§Retinoblastoma occurs with complete penetrance in this single line of mice (Windle et al., 1990).

**Table 3. Transgenic mice carrying the rat LH $\beta$  promoter linked to T antigen**

Mouse line	Pituitary tumors*	Ectopic tumors†	Reproductive status‡
rLT1	none	brain	infertile
rLT2	none		no transmission
rLT3	none	brain	no transmission
rLT4	occasional 100-200 d	brain, pancreas	M fertile, F infertile
rLT5	none	brain, pancreas	fertile
rLT6	none	pancreas	infertile

\*Animals were killed or died due to pituitary or other tumors at the age indicated.

†Tumors, other than pituitary tumors, that were identified upon autopsy.

‡Indicates the fertility profile of the founder and transgenic lines. (M) male, (F) female.

expression in the hLT mice was similar to the  $\alpha$ T mice in that  $\alpha$ -subunit was expressed in all tumors (Fig. 3A). However, in contrast to the  $\alpha$ T tumors, hLT tumors also expressed the glycoprotein hormone  $\beta$ -subunits. The tumor dissected from animal hLT1-3 expressed both TSH $\beta$  and LH $\beta$ , and that of animal hLT15-10 expressed high levels of LH $\beta$  and FSH $\beta$ .

In contrast to the hLT mice, those animals generated using the rat LH $\beta$  5' flanking sequence lacked pituitary-specific T antigen expression, developing tumors more frequently in the brain and the pancreas (rLT mice, Table 3). Ectopic tumor formation in the brain and pancreas are common in T antigen transgenic mice due to 'leaky' T antigen expression in these tissues (Brinster et al., 1984). One transgenic mouse line, rLT4, occasionally developed pituitary tumors which were manifested at an early age of 100-200 days, but this phenotype was not uniformly inherited. Tumors derived from the rLT4 mouse line expressed high levels of three gonadotropin subunits but not TSH $\beta$  (Fig. 3B). (The lower band seen in the blot hybridized with an FSH $\beta$  probe is residual  $\alpha$ -subunit probe which was inadequately stripped.) As was the case for the  $\alpha$ T tumors, GH and POMC mRNA were not expressed but Prl mRNA was expressed to varying degrees in the tumors from the hLT animals (data not shown).

Since the human LH $\beta$  5' region targeted the pituitary with fidelity, while a longer fragment of the rat LH $\beta$  5' DNA failed to demonstrate specificity, it is clear that the species derivation of the promoter used to drive T antigen expression can be an important variable in the determination of the type of tumors formed. Furthermore, the appearance of FSH $\beta$  mRNA in some of the hLT and rLT tumors may signal the presence of fully differentiated gonadotropes within the tumor cell population.

To target T antigen-mediated immortalization to the  $\alpha$ -subunit-expressing bipotential progenitor cells that exist prior to the branching of the thyrotrope and gonadotrope lineages in the pituitary anlage, we used a transgene that contained 5.5 kb of the human  $\alpha$ -subunit regulatory region linked to the coding region of T antigen. This strategy was based on the information that the 1.8 kb fragment of the human gene directed T antigen (Windle et al., 1990) and  $\beta$ -galactosidase (Hamernik et al., 1992) expression only to the gonadotrope. Furthermore, a 4.6 kb fragment of the mouse  $\alpha$ -subunit gene was recently used to direct expression of  $\beta$ -galactosidase to both the thyrotrope and gonadotrope lineages (Kendall et al., 1994) suggesting that sequences upstream may be required to target the

**Table 4. Transgenic mice carrying the 5.5 kb human  $\alpha$ -subunit promoter linked to T antigen**

Mouse line	Pituitary tumors*	Ectopic tumors†	Reproductive status‡
5 $\alpha$ T12	67 d	brain	M fertile; F infertile
5 $\alpha$ T17	none	choroid plexus, 67 d	infertile
5 $\alpha$ T40	homozygote, 197 d	bladder, liver, 253 d	fertile
5 $\alpha$ T45	165 d		kidney, stomach fertile

\*Animals were killed or died due to pituitary or other tumors at the age indicated.  
†Tumors in addition to pituitary tumors which were identified upon autopsy.  
‡Indicates the fertility profile of the founder and transgenic lines. (M) male, (F) female.

thyrotrope lineage. Four transgenic mouse lines were generated using 5.5 kb of the human  $\alpha$ -subunit gene linked to T antigen. Mice from three of these transgenic lines develop pituitary tumors at various ages (5 $\alpha$ T mice, Table 4). Pituitary tumors in the 5 $\alpha$ T12 line are the most aggressive resulting in female infertility and fatality by 67 days of age. Both sexes of lines 5 $\alpha$ T40 and 5 $\alpha$ T45 are fertile and were bred to homozygosity to accelerate tumor formation. Northern blot analysis of pituitary tumor RNA shows that the  $\alpha$ -subunit gene is expressed to varying degrees in the pituitary tumors from the three transgenic mouse lines (Fig. 4). Unlike the  $\alpha$ T tumors created with the 1.8 kb fragment of the human gene, some of the 5 $\alpha$ T tumors expressed the  $\beta$ -subunits of the gonadotropin hormones. Interestingly, however, the expression of the  $\beta$ -subunits was inconsistent between tumors even from the same transgenic mouse line. For example, Fig. 4 illustrates that, while some tumors analyzed from line 5 $\alpha$ T45 expressed the  $\beta$ -subunit of LH and/or FSH (5 $\alpha$ T45-3), other tumors taken from animals of the same line did not (5 $\alpha$ T45-6). This inconsistency points out that not all tumors generated from a single transgenic mouse line are identical and that they may arise from multiple independent clonal events within each tumor. Though this transgene might have been expected to target both the thyrotrope and gonadotrope lineages, none of these tumors expressed detectable levels of the  $\beta$ -subunit of TSH.

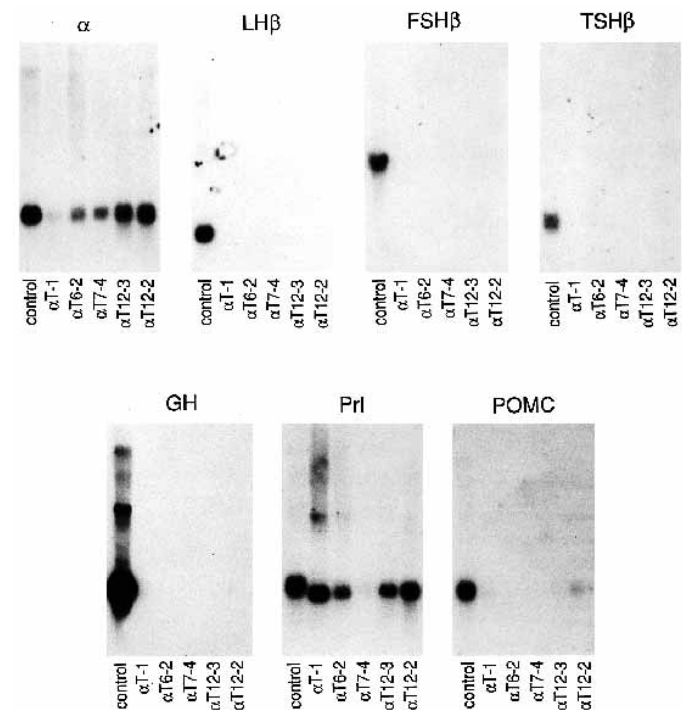
#### Cell lines derived from the pituitary tumors reflect various stages of pituitary cell differentiation

Tumors isolated from many of the transgenic lines were dispersed and maintained in culture for several months to free the immortalized cells (those that express T antigen) from supportive connective tissue, blood cells, endothelial cells, and nontransformed pituitary cells. Three novel cell lines were derived from tumors of the rLT and 5 $\alpha$ T transgenic mouse lines. Cell lines could not be derived from the hLT mice since the tumors grow slowly and are not manifested until the animals are over 1 year old. In several attempts, we have not been successful at generating cell lines from tumors taken from animals older than 10 months of age (see Table 2). Once subclones were isolated, the expression pattern of pituitary hormones in the individual cell lines was analyzed and compared to the primitive gonadotrope cell line,  $\alpha$ T3-1, to identify the lineages and developmental stages targeted by the respective promoters (Fig. 5). None of the cell lines generated

express markers of the corticotrope, somatotrope or lactotrope lineages (Fig. 5).

The L $\beta$ T4 cell line was generated from pituitary tumors that developed in transgenic animals of the rLT4 line in which T antigen is under the control of the rat LH $\beta$ -subunit promoter. As predicted from the expression pattern in the tumor, the L $\beta$ T4 cells express the  $\beta$ -subunit of LH in addition to  $\alpha$ -subunit and GnRH receptor (GnRH-R), the gonadotrope genes expressed by the  $\alpha$ T3-1 cell line (Fig. 5). L $\beta$ T4 and a second cell line derived from a tumor in another mouse from the same transgenic line (L $\beta$ T2; data not shown) are the only cell lines that express the more differentiated gonadotrope marker, the LH $\beta$ -subunit gene. They do not express the  $\beta$ -subunit of FSH (Fig. 5), and therefore are most likely to represent a narrow window in pituitary gonadotrope development between the onset of LH $\beta$ -subunit (E16.5) and FSH $\beta$ -subunit (17.5) gene expression.

In accordance with our original hypothesis that the 5.5 kb fragment of the human  $\alpha$ -subunit gene might target expression to an earlier stage of pituitary development, we isolated a primitive cell, the  $\alpha$ T1-1 cell line, that expresses the  $\alpha$ -subunit gene but does not express any further differentiated markers of the anterior pituitary lineages (Fig. 5). The  $\alpha$ T1-1 cell line was isolated from a tumor from the 5 $\alpha$ T12 mouse line. Several cell lines generated from the 5 $\alpha$ T45 transgenic mouse line were of the gonadotrope lineage and were similar to the  $\alpha$ T3-1 cell



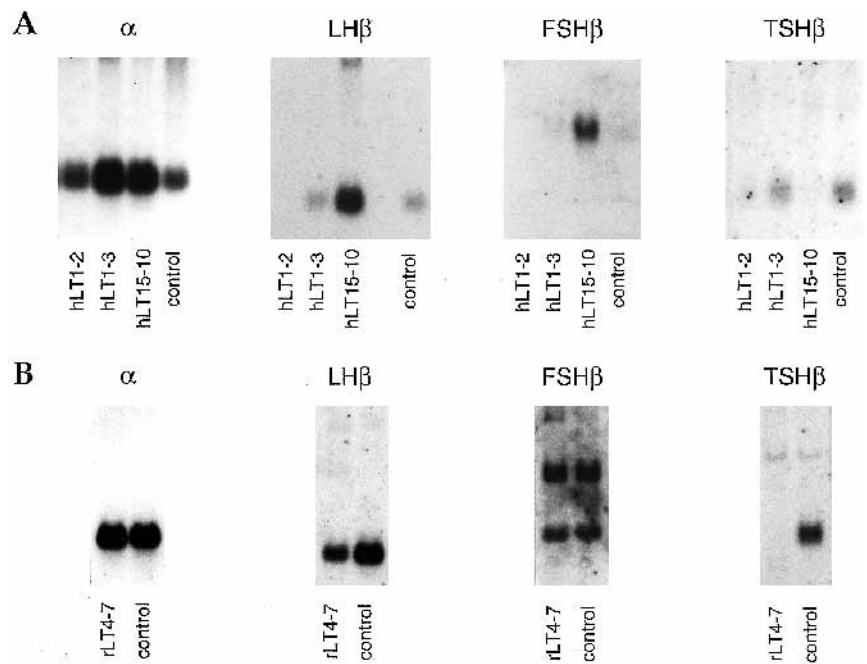
**Fig. 2.** Hormone mRNA expression in the tumors from the  $\alpha$ T transgenic mice. 10  $\mu$ g of total RNA from pituitary tumors from  $\alpha$ T mice were analyzed by northern blot for the expression of anterior pituitary hormones relative to a normal control pituitary. This figure represents a single blot that was hybridized and stripped sequentially with the followings probes: mouse  $\alpha$ -subunit (Chin et al., 1981), rat LH $\beta$  (Tepper and Roberts, 1984), rat FSH $\beta$  (Maurer, 1987), mouse TSH $\beta$  (Gurr et al., 1983), rat GH (Seeburg et al., 1977), rat PRL (Cooke et al., 1980) and rat POMC (Eberwine and Roberts, 1984).

line, while others resembled the  $\alpha$ T1-1 cell line (data not shown).

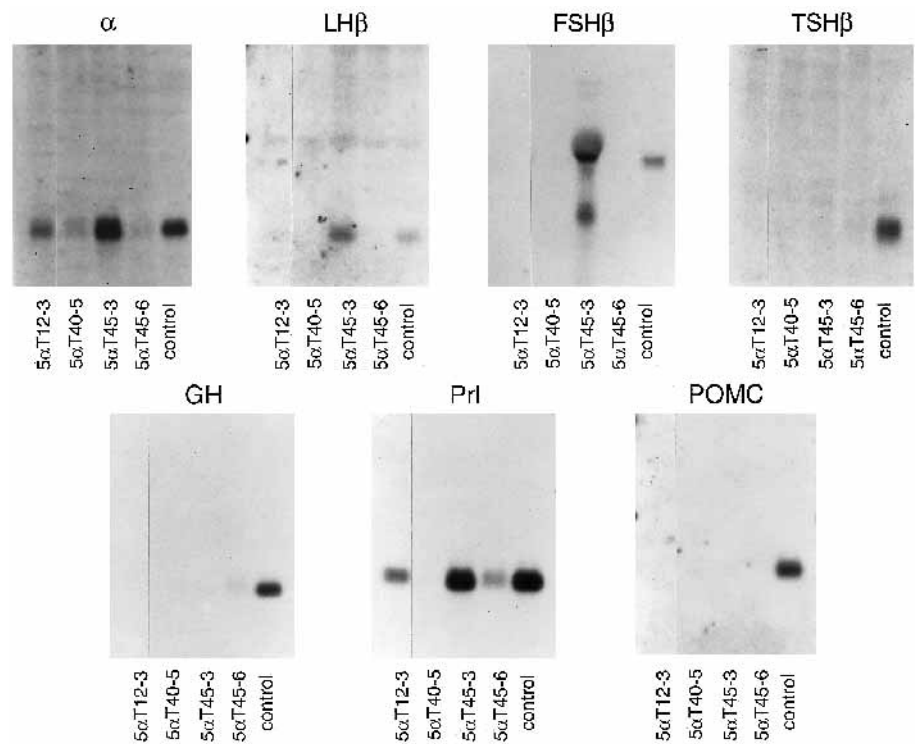
Though we did not detect TSH $\beta$  gene expression in the 5 $\alpha$ T pituitary tumor fragments that we analyzed, we were able to isolate a single TSH $\beta$ -expressing cell line, the T $\alpha$ T1 cell line (Fig. 5), from a tumor of the 5 $\alpha$ T12 transgenic line. One possible explanation for the presence of thyrotropes is that two independent clonal transformation events may have taken place in the same pituitary giving rise to two cell types present at different levels or locales within the same tumor. Alternatively, since TSH $\beta$ -subunit initiates 3 days after  $\alpha$ -subunit expression, one might predict that a population of immortalized primitive  $\alpha$ -subunit expressing cells was present prior to the immortalization of a differentiated thyrotrope. If this were the case, the majority of the tumor would be composed of the primitive cell type that gave rise to the  $\alpha$ T1-1 cell line and the thyrotrope cells that gave rise to the T $\alpha$ T1 cell line would be in the minority and below the sensitivity of the assay. However, more sensitive detection methods, such as RT-PCR and immunocytochemistry, would likely confuse the interpretation of tumor data because of possible contamination with normal pituitary cells.

#### Immortalized cell lines express pituitary transcription factors

The individual endocrine cell types and lineages of the anterior pituitary are marked by the expression of specific transcription factors as well as hormone subunits and receptors. Recent studies have identified two factors from the lim homeodomain family, mLIM-2 and mLIM-3 (also termed LH-2 and P-lim, respectively) that are expressed in the fetal and adult anterior pituitary (Bach et al., 1995; Roberson et al., 1994; Seidah et al., 1994). These two factors are expressed during active differentiation in the fetal pituitary and may be important in transcriptional activation of gene expression in several anterior pituitary cell types. Therefore, we tested for their expression in the precursor ( $\alpha$ T1-1), thyrotrope (T $\alpha$ T1) and two gonadotrope cell lines ( $\alpha$ T3-1 and L $\beta$ T4) (Fig. 6). Both mLIM-3 and mLIM-2 were expressed in all four cell lines indicating that expression of these factors does not correlate with the transitions during thyrotrope and gonadotrope differentiation



**Fig. 3.** Glycoprotein hormone subunit mRNA expression in tumors created by the LH $\beta$  promoter/T antigen transgenes. 10  $\mu$ g of total RNA from pituitary tumors from (A) hLT and (B) rLT transgenic mice were analyzed for the expression of glycoprotein hormone subunit mRNAs relative to a normal pituitary control. Northern blots were sequentially hybridized with the following cDNA probes: mouse  $\alpha$ -subunit, rat LH $\beta$ , rat FSH $\beta$ , mouse TSH $\beta$  (as in Fig. 2).



**Fig. 4.** Analysis of pituitary hormone mRNA expression in 5 $\alpha$ T transgenic mouse tumors. 10  $\mu$ g of total RNA from pituitary tumors from 5 $\alpha$ T mice were analyzed by northern blot for the expression of anterior pituitary hormones relative to a normal control pituitary. Northern blots were sequentially hybridized with the following cDNA probes: mouse  $\alpha$ -subunit, mouse LH $\beta$ , rat FSH $\beta$ , mouse TSH $\beta$ , rat GH, mouse PrL and rat POMC (as in Fig. 2).

represented by these cell lines and that they are likely to be active prior to the activation of the  $\alpha$ -subunit gene.

Another murine homeobox gene localized to Rathke's pouch, *Rpx*, is the earliest known transcriptional regulator detected in this embryonic tissue (Hermesz et al., 1996). *Rpx* expression is observed as early as E9 in the layer of ectodermal cells that will give rise to Rathke's pouch and is progressively lost from E12.5 to E15.5, correlated with the emergence of the differentiated endocrine cell types. Northern blots using an *Rpx* cDNA probe (kindly provided by K. Mahon) have thus far failed to detect *Rpx* expression in the RNA from these cell lines, perhaps indicating that *Rpx* expression is lost prior to commitment to differentiated cell fate in the gonadotrope and thyrotrope lineages.

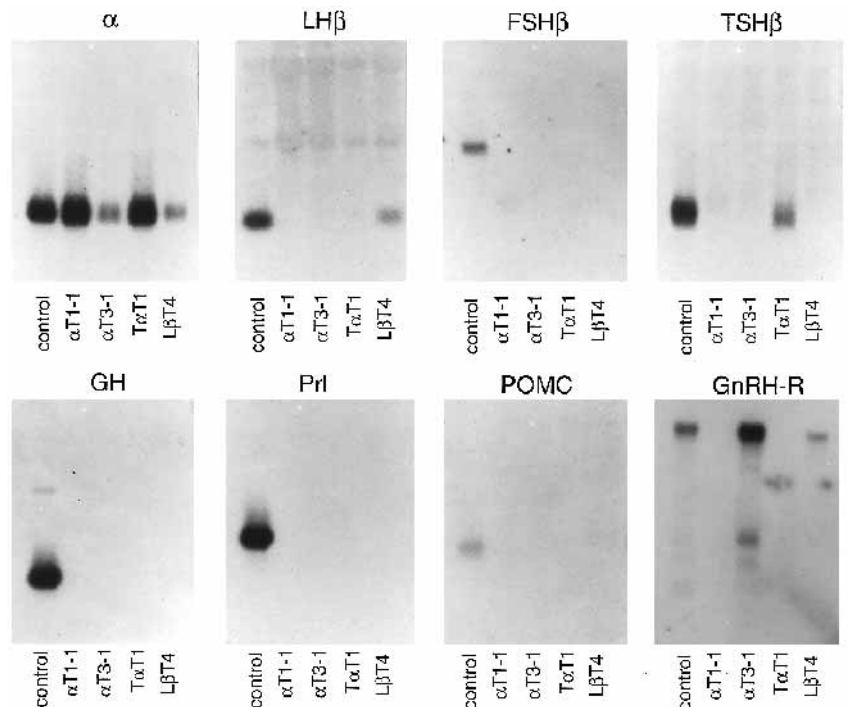
### Pituitary cell lines produce cell type-specific transcription factors

Barnhart and Mellon (1994) demonstrated that the transcription factor, steroidogenic factor (SF-1), regulates the  $\alpha$ -subunit gene in the  $\alpha$ T3-1 cell line through a gonadotrope specific element ( $\alpha$ GSE) and that its expression is restricted to gonadotrope cell lines. Moreover, Ingraham et al. (1994) determined that expression of SF-1 colocalizes with LH $\beta$  and FSH $\beta$  expression in the pituitary, initiating at E13.5, subsequent to  $\alpha$ -subunit expression. To test whether SF-1 expression precedes the expression of other differentiated markers of the gonadotrope lineage (i.e. GnRH receptor), the cell lines were assayed for SF-1 expression with particular interest in the  $\alpha$ T1-1 cell line. Appropriately, SF-1 is expressed in both of the  $\alpha$ T3-1 and L $\beta$ T4 gonadotrophic cell lines but was not detectable in either the TSH-expressing T $\alpha$ T1 thyrotropic cells or the early  $\alpha$ T1-1 early progenitor cells (Fig. 7A). Upon screening multiple  $\alpha$ -subunit-expressing cell lines, we have yet to find one that expresses SF-1 in the absence of GnRH receptor or vice versa, suggesting that these genes may share common regulatory components.

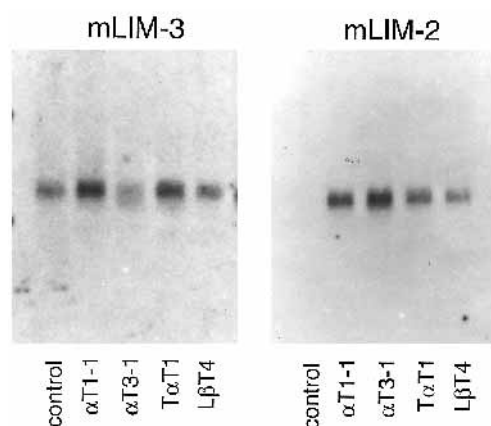
To demonstrate the gonadotrope specificity of the SF-1 protein, we utilized the  $\alpha$ GSE as a probe in electrophoretic mobility shift assays (EMSAs) with nuclear proteins (Fig. 7B). Specific protein complexes bind to this element in nuclear extracts from all cell lines as determined by competitions with unlabeled probe and a mutant  $\alpha$ GSE (mGSE) probe. However, an SF-1 competitor oligonucleotide derived from -50 to -36 of the human cholesterol side-chain cleavage enzyme gene (Rice et al., 1990) is only capable of displacing the specific complex in the  $\alpha$ T3-1 and L $\beta$ T4 gonadotrope cells, demonstrating that this major complex is SF-1.

The POU-homeodomain protein GHF-1 (Pit-1) is crucial for the maintenance of mature somatotropes, lactotropes and thyrotropes in vivo (Li et al., 1990). Yet the onset of TSH $\beta$  expression precedes GHF-1 by at least one day (Simmons et al., 1990) and may be present in two populations of cells, an early

transient population present in the developing pars tuberalis of the anterior pituitary that does not express GHF-1 and a later population arising in the pars distalis that is dependent on GHF-1 for survival and persists through adulthood (Lin et al., 1994). Therefore, to further delineate the identity and developmental stage of the thyrotropic T $\alpha$ T1 cells, northern blots were performed using a radiolabeled GHF-1 cDNA probe and EMSAs were performed with a probe corresponding to a consensus GHF-1 DNA-binding site (Fig. 8). The T $\alpha$ T1 cells expressed two transcripts of the approximate size of 2.6 kb and 1.9 kb that comigrate with transcripts in the normal mouse pituitary (Fig. 8A). However, evidence exists that the onset of GHF-1 transcription may precede the appearance of GHF-1 protein (Dollé et al., 1990). Furthermore, it has been suggested that GHF-1 is translationally controlled in the  $\alpha$ TSH cell line (Gordon et al., 1993), a thyrotropic cell line that expresses  $\alpha$  subunit but not TSH  $\beta$ -subunit (Akerblom et al., 1990). EMSA demonstrates that nuclear extracts from the T $\alpha$ T1 cells contain a protein that binds a consensus GHF-1-binding site with an identical pattern to extracts from the GHFT-1 cell line that expresses GHF-1 (Lew et al., 1993). This specific complex was displaced from the binding site by 100-fold excess of unlabeled GHF-1 oligonucleotide but not unlabeled SF-1 oligonucleotide (Fig. 8B). GHF-1 binding was not observed in an EMSA performed with  $\alpha$ T1-1,  $\alpha$ T3-1 or L $\beta$ T4 nuclear protein extracts (data not shown). These data indicate that the thyrotropic T $\alpha$ T1 cell line is most likely derived from the surviving pars distalis



**Fig. 5.** Pituitary hormone mRNA expression in cell lines derived from transgenic tumors. 10  $\mu$ g of total RNA, isolated from a normal pituitary control and the indicated cell lines, was analyzed by northern blot for the expression of anterior pituitary hormones and gonadotropin-releasing hormone receptor (GnRH-R). Northern blots were sequentially hybridized with radiolabeled cDNA probes for anterior pituitary cell lineage markers as in Fig. 2. Note that the  $\alpha$ T3-1 and L $\beta$ T4 cell lines express gonadotrope-specific markers and the T $\alpha$ T1 cells express thyrotrope-specific markers.



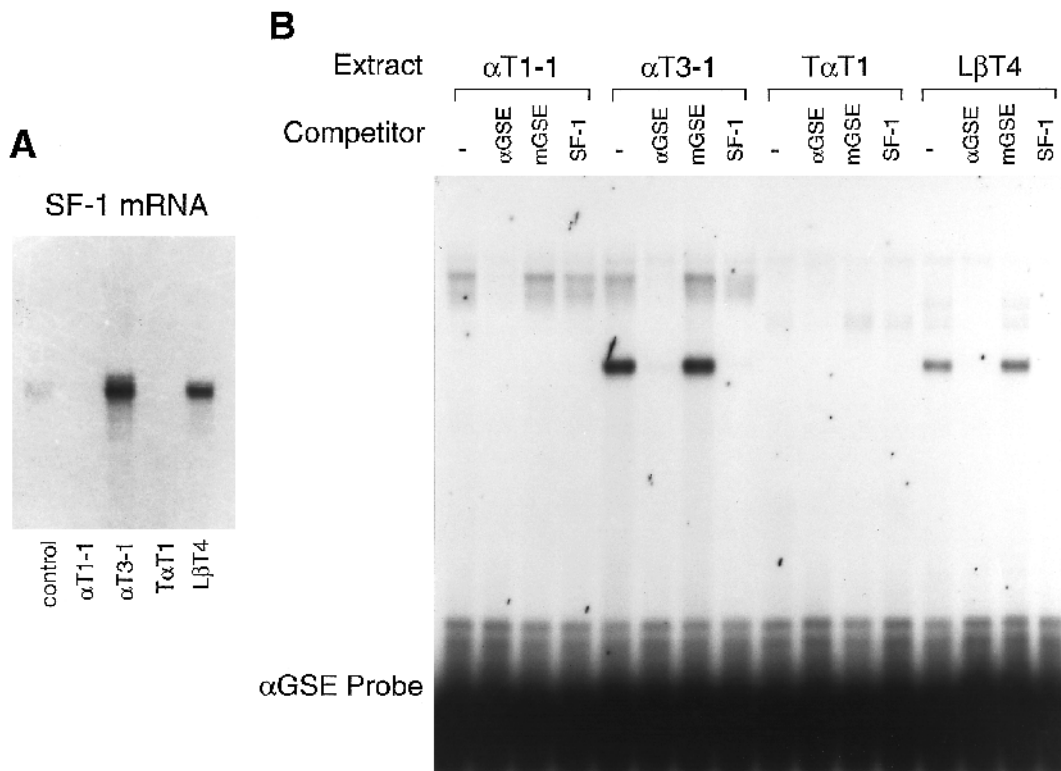
**Fig. 6.** Expression of lim homeodomain transcription factors. The indicated cell lines were analyzed for the expression of two lim-homeodomain transcription factor family members, mLIM-3 and mLIM-2 by northern blot analysis. The cDNA probe for mLIM 2 was generously provided by Dr Tom Jessell and Dr Fred Alt (Xu et al., 1993). The cDNA used for the mLIM-3 probe is a PCR-derived sequence cloned from mouse anterior pituitary cDNA using primers encompassing the entire coding sequence (Seidah et al., 1994). Though expression was detected in all four cell lines, mLIM-2 could not be detected in total RNA from the adult pituitary and may be present below the level of detection.

thyrotrope cell population that is dependent on GHF-1 and persists in adult pituitary.

**Fig. 7.** Gonadotrope cell lines produce SF-1. (A) Northern blot analysis of total RNA (10 µg) hybridized with a cDNA probe for steroidogenic factor-1 (SF-1) (Barnhart and Mellon, 1994). Note that only the control normal pituitary and the two gonadotrope cell lines (αT3-1 and LβT4) express SF-1 mRNA.

(B) Nuclear protein extracts from the various cell lines were analyzed for DNA-binding activity with a radiolabeled double-stranded oligonucleotide probe of the gonadotrope-specific element in the human α-subunit promoter (αGSE). Assays were performed as described in the Materials and Methods. The specificity of the complexes was verified by competing with 100-fold excess of cold competitor oligonucleotides as designated (mGSE indicates a mutated αGSE, see Methods and Materials). Note that

while all four extracts contained specific DNA-binding proteins, only extracts from the gonadotrope cell lines (αT3-1 and LβT4) formed complexes that were selectively inhibited when an SF-1 site from the human cholesterol side-chain cleavage enzyme gene (Rice et al., 1990) was used as a competitor.

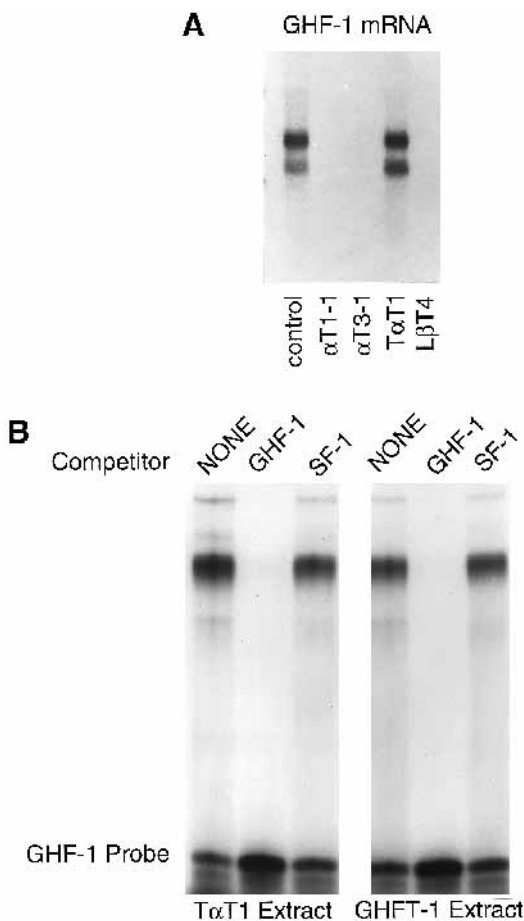


## DISCUSSION

Targeted tumorigenesis in transgenic mice has been a powerful tool for the study of oncogenesis and gene expression, and for the production of differentiated immortal cell lines from particular tissues (Camper, 1987; Hanahan, 1989). Here we demonstrate that immortalization can be targeted not only spatially but temporally as well. This approach allows the generation of cell lines representative of discrete stages of development within cell lineages that occur transiently within complex tissues and would otherwise be inaccessible. In this study, we utilized 5' flanking regions of genes expressed at definitive stages of commitment along the differentiation pathway of the gonadotrope and thyrotrope cell lineages to direct oncogene expression. In each case, this initiated a transformation process that immortalized cells at the stage when the regulatory regions contained within the respective upstream sequences become active (Fig. 9). The resulting cells maintain expression of the set of differentiated markers of the cell lineages characteristic of the stage of differentiation at which the targeting gene is first activated during development.

Based on our previous results in which immature gonadotrope cell lines were generated using 1.8 kb of the human α-subunit promoter to direct Tag expression (Windle et al., 1990), we hypothesized that it might be possible to immortalize cells that represented intermediate steps in the differentiation pathway of a given cell lineage by directing T antigen expression with upstream regulatory sequences of markers that are activated at sequential stages of development. The glyco-





**Fig. 8.** The T $\alpha$ T1 cell line is a differentiated thyrotrope cell that produces GHF-1 (Pit-1). (A) 10  $\mu$ g of total RNA isolated from the various cell lines were analyzed for the expression of GHF-1. The cDNA probe used was a fragment of the rat GHF-1 coding sequence (Bodner et al., 1988). (B) Extracts from the T $\alpha$ T1 were assayed by EMSAs for the ability to form specific complexes with a GHF-1 consensus binding site. Extracts from the GHF-1 overexpressing cell line, GHFT-1 (Lew et al., 1993), were analyzed in the same assay as a positive control. Competitions were performed using 100-fold excess of cold probes of the GHF-1- and SF-1-binding sites verifying the specificity of the shifted complexes.

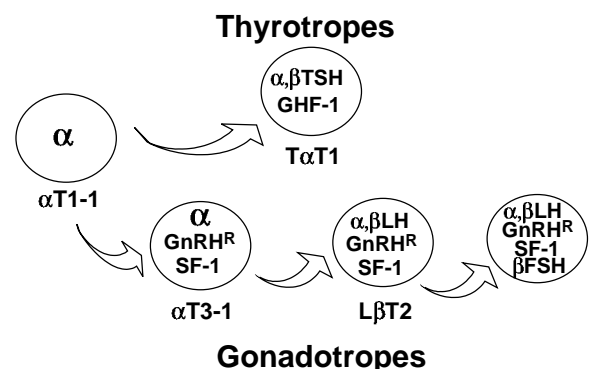
protein lineage of the anterior pituitary proved to be an excellent model in which to test this hypothesis since multiple stages of its differentiation pathway are distinguished by the expression of individual subunit genes allowing us to target expression to intermediate stages in development and to identify characteristic marker genes expressed in the resultant cell lines.

The initiation of anterior pituitary differentiation is marked by the expression of the glycoprotein hormone  $\alpha$ -subunit gene. Subsequently,  $\alpha$ -subunit-expressing cells commit to either the thyrotrope or gonadotrope lineage. The mature thyrotropes arise first on E14.5 with the expression of TSH $\beta$ -subunit, while the emergence of mature gonadotropes occurs in two stages – with the expression of LH $\beta$ -subunit gene and FSH $\beta$ -subunit gene on E16.5 and E17.5, respectively. Based on this temporal pattern of expression of individual subunit genes, 5' flanking sequences were chosen that when linked to T antigen were

likely to immortalize cells that developmentally flank the original  $\alpha$ T3-1 pregonadotrope cell line. Using 1.8 kb of the rat LH $\beta$  promoter and 5.5 kb of the human  $\alpha$ -subunit promoter, we successfully immortalized cells that represent multiple steps of the above defined differentiation pathway.

As predicted, targeting oncogenesis with the 5' flanking region of the LH $\beta$  gene produced cell lines that exhibit many of the highly specific characteristics of a mature gonadotrope, with the exception of the expression of FSH $\beta$  gene. In addition to expressing both the LH $\alpha$  and LH $\beta$  subunits, the GnRH receptor and the transcription factor SF-1, the L $\beta$ T4 and L $\beta$ T2 cell lines secrete intact, biologically active LH (Turgeon et al., 1996). This response is strongly augmented by estrogen, dexamethasone and pulsatile administration of GnRH (Turgeon et al., 1996). Furthermore these cells demonstrate intracellular calcium fluxes in response to GnRH indicating fidelity of gonadotrope physiological behavior (Thomas et al., 1996).

The 5.5 kb  $\alpha$ -subunit regulatory region linked to T antigen caused immortalization of cell lines representative of  $\alpha$ -subunit-expressing cells that are present prior to the commitment to either glycoprotein lineage or are present subsequent to the branching of the gonadotrope and thyrotrope lineages. This suggests that elements necessary for thyrotrope-specific targeting and for early expression of  $\alpha$ -subunit in a bipotential progenitor reside between  $-5.5$  kb and  $-1.8$  kb of the human gene, upstream of gonadotrope-specific elements. This presumptive enhancer likely contains both cell-specific as well as temporal regulatory sequences. These results emphasized that, while 1.8 kb of the 5' flanking region is sufficient to direct oncogene expression to the gonadotrope lineage, it does not contain the regulatory elements necessary for  $\alpha$ -subunit expression in the anterior pituitary anlage or in the thyrotrope lineage (see also Hamernik et al., 1992). These observations are in agreement with Kendall et al. (1994), who, using varying lengths of 5' flanking region of the mouse  $\alpha$ -subunit linked to the reporter gene,  $\beta$ -galactosidase, demonstrated that while 0.48 kb was sufficient to direct minimal expression to both the gonadotrope and thyrotrope cells in transgenic mice, sequences between  $-4.6$  and  $-2.7$  conferred high levels of reporter gene expression in the thyrotrope lineage. This enhancer in the



**Fig. 9.** The gonadotrope and thyrotrope cell lines fall along the developmental cell lineages of the anterior pituitary. A diagrammatic representation of the cell lineages for the thyrotrope and gonadotrope lineages illustrates the distinct stages of differentiation represented by the immortalized pituitary cell lines created by targeted oncogenesis in transgenic mice. GnRH receptor (GnRH<sup>R</sup>).

mouse gene was not detectable using in vitro assays. Future studies will attempt to delineate enhancer elements upstream of -1.8 kb in the human  $\alpha$ -subunit gene.

Surprisingly, in addition to the progenitor cell expressing only the  $\alpha$ -subunit gene, a thyrotrope cell was derived from tumors in which TSH $\beta$ -subunit could not be detected by northern blot. This cell expresses the transcription factor GHF-1 and responds to thyroid hormones (B. Yusta, personal communication) and thus represents a mature thyrotrope. Similar attempts to direct transgene expression to the thyrotrope lineage were unsuccessful using the mouse TSH $\beta$  promoter (David Gordon, personal communication). The only successful targeting with a TSH $\beta$  promoter generated pituitary tumors that were completely undifferentiated (Maki et al., 1994). Thus, in this case, using sequences upstream of -1.8 kb of the human  $\alpha$ -subunit gene (which drive expression in both thyrotropes and gonadotropes) proved to be more successful than using the cell type-specific TSH $\beta$  promoter to immortalize mature thyrotrope cells.

The question remains as to whether the immortalization event occurs during development, freezing the cells in a discrete stage of differentiation, or whether the transformation event occurs in more differentiated cells, which then dedifferentiate during tumor formation. The dedifferentiation process would be limited by the fact that expression of the oncogene is controlled by regulatory region from genes expressed only in differentiated cells. Thus, dedifferentiation beyond the point at which the regulatory region is active would result in cessation of cell division and loss from the tumor or cell culture. It is unlikely that dedifferentiation occurs only during culturing, since the pattern of expression in the cultured cells correlates with that of the original tumors. Most likely, the final phenotype of the cell lines derived results from a combination of these events. Regardless, it is clear that directed oncogenesis results in cells that mirror the discrete stage of development at which the targeting promoter is first activated.

The availability of somato/lactotrope and corticotrope cell lines have contributed substantially to our understanding of the molecular events required for activation of the genes that define their respective cell lineages. Specifically, the use of GH3 and GC cells that express GH and Prl, and the AtT20 cells that express POMC, led to the discovery of the POU-homeodomain protein, GHF-1/Pit-1 (Bodner et al., 1988; Ingraham et al., 1988), and the helix-loop-helix transcription factor family member, CUTE (corticotrope upstream transcription element-binding) protein (Therrien and Drouin, 1993). These transcription factors activate expression of the genes that mark critical stages in the commitment towards a differentiated phenotype (Bodner et al., 1988; Dollé et al., 1990; Ingraham et al., 1988; Mangalam et al., 1989; Therrien and Drouin, 1993). In fact, GHF-1 is required for the development and maintenance of the somatotrope, lactotrope and thyrotrope cell lineages since dwarf mice in which the GHF-1 gene is mutated or deleted fail to develop these cell types (Li et al., 1990).

The derivation of the  $\alpha$ T3-1 cells permitted the dissection of regulatory elements required for gonadotrope-specific expression of the glycoprotein  $\alpha$ -subunit gene (Horn et al., 1992) and led ultimately to the finding that the nuclear orphan receptor SF-1 contributes to the its gonadotrope-specific expression (Barnhart and Mellon, 1994). In this study, we further demonstrate that SF-1 is expressed only in cells that are

committed to the gonadotrope lineage. Although SF-1 may not be required for the differentiation of the gonadotrope (Ikeda et al., 1995); our data, in conjunction with the known timing of its developmental onset (Ingraham et al., 1994), suggest that the expression of SF-1 is an early marker of the gonadotrope lineage. Furthermore, the high level of expression of the GnRH receptor by  $\alpha$ T3-1 cells, permitted the cloning of the cDNA for this key receptor (Tsutsumi et al., 1992). With the addition of the  $\alpha$ T1-1, L $\beta$ T4, L $\beta$ T2 and T $\alpha$ T1 cell lines, we now have the tools to further investigate the molecular mechanisms governing gonadotrope- and thyrotrope-specific gene expression as well as the molecular events that confer cell identity during differentiation and that control cell fate decisions. Our results demonstrate the potential for immortalization of a series of developmental progenitor cells using the regulatory regions from cell-type-specific genes, an approach that may prove to be uniquely suited to the study of mammalian organogenesis.

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