Variegated Expression of a Mouse Steroid 21-Hydroxylase/β-Galactosidase Transgene Suggests Centripetal Migration of Adrenocortical Cells

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5′-Flanking sequences (6.4 kb) of the mouse steroid 21-hydroxylase (21-OHase) A gene linked to a LacZ reporter gene directed appropriate cell-specific expression in cultured Y1 adrenocortical tumor cells and in the adrenal cortex of transgenic mice. The transgene expression initiated at the same stage of adrenal development as the endogenous 21-OHase gene (embryonic day 11.5). Although the endogenous 21-OHase gene is expressed throughout the adrenal cortex, the 21-OHase/β-gal transgene showed a strikingly variegated pattern of adrenocortical expression in all 10 transgene-expressing mouse lines examined. This presents as radial stripes of β-gal staining transcending the classical zonal structure of the adrenal cortex but paralleling the columnar arrangement of cells of the zona fasciculata and the centripetal organization of the adrenocortical blood supply. To the extent that the variegated pattern of 21-OHase/β-gal transgene expression depicts adrenocortical cell lineage, these results suggest that all cells within an individual stripe have a common clonal origin; the radial pattern of clonally derived cells argues that cellular migration maintains the adult adrenocortical cell population. Adrenal glands of developing embryos also exhibited a variegated pattern of 21-OHase/β-gal transgene expression. However, this presented as islands of β-gal reporter staining within the developing gland, suggesting that the rapid embryonic adrenal growth phase, which precedes the establishment of the classic adrenocortical zonal structure, may be governed by cellular mechanisms distinct from those responsible for maintenance of the adult adrenocortical cell population. (Molecular Endocrinology 10: 585–598, 1996)

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INTRODUCTION

The adrenal cortex is a major endocrine source of circulating steroid hormones, these being synthesized from cholesterol by the sequential actions of a related group of cytochrome P450 enzymes (1). Some of these enzymes are expressed exclusively in the adrenal gland, while others are present in all steroidogenic tissues. The adult adrenal cortex is classically divided into three concentric, morphologically distinct zones, the outer zona glomerulosa, the zona fasciculata, and the inner zona reticularis, defined by their different cellular arrangements (2). These zones are also functionally distinct, *i.e.* mineralocorticoids are synthesized in the zona glomerulosa, whereas glucocorticoids are produced by the zona fasciculata/reticularis (1). In humans and some other mammals, androgenic steroids are synthesized in the zona reticularis, although this is not the case in rodents. The zona reticularis is less prominent in these species, but it remains the major location of cell death in the adrenal cortex (3, 4). A second level of adrenocortical organization is apparent from the centripetal arrangement of the adrenal blood supply: arteries to the outer capsule give rise to capillaries that cross the cortex at right angles to the concentric zonal arrangement, ultimately uniting at the cortical-medullary boundary (2). Furthermore, the cells of the zona fasciculata are arranged in columns that extend from the zona glomerulosa to the zona reticularis, emphasizing that adrenocortical organization may not necessarily follow morphological zonation.

The adrenal cortex is a dynamic organ in which senescing cells are constantly replaced by newly differentiated daughter cells (5). Both proliferation within a zone (the zonal theory) (6) and cell migration (7) have been suggested to explain cytogenesis in the adrenal cortex (reviewed in Refs. 2 and 5). The former proposes that each zone maintains its own cell population by proliferation within the zone and was favored when it became clear that the different morphological zones of the cortex were also functionally distinct. However, autonomy of the zones is difficult to reconcile with the observed distribution of mitoses and cell death that predominate, respectively, in the zona glomerulosa and the zona reticularis of the adrenal cortex (3, 4, 8–10). The cellular migration theory suggests that cell proliferation occurs mainly at the periphery of the cortex and that existing cells are displaced centripetally through the fasciculata to the reticularis where they are finally eliminated. In this scheme, all cells of the adrenal cortex have a common origin, and their functional identity at a given time is determined by their immediate environment. It is thus implied that the phenotype of an individual adrenocortical cell changes during its lifetime. Such a scheme has profound implications for the control and regulation of genes that are differentially expressed in the adrenal zones, especially the genes of the adrenal steroidogenic pathways. Most of the data supporting centripetal migration of cells have been obtained from rapidly growing tissue seen in early postnatal stages, or after surgical intervention (2, 11). It is less clear whether centripetal migration is a feature of the mature adrenal gland, in which mitosis is balanced by cell death, or by what means the zonal organization of the adrenal cortex is established during embryogenesis. To understand the mechanisms that govern differential gene expression between the adrenocortical zones and their distinct functions, in both normal and disease states, it would be essential to distinguish between zonal and migratory modes of adrenocortical maintenance.

The steroid 21-hydroxylase (21-OHase, Cyp21) gene is normally expressed throughout the adrenal cortex (12), where it mediates the penultimate step in both glucocorticoid and mineralocorticoid synthesis (1). In this study, we assessed the extent to which the cloned promoter region of 21-OHase gene is able to recapitulate the adrenocortical-specific expression pattern of its endogenous counterpart when linked to a LacZ reporter gene, and thus whether this promoter can be used to direct heterologous gene expression to specific adrenal cell types. We show that the 21-OHase promoter/β-galactosidase (21-OHase/β-gal) reporter transgene, is expressed specifically in the adrenal cortex of transgenic mice. However, in contrast to the pan-cortical expression pattern of the endogenous 21-OHase gene, the LacZ reporter transgene displays a strikingly variegated pattern of expression, which presents as radial stripes of β-galactosidase staining in the adult adrenal cortex of all 10 transgene-expressing lines examined. This restriction of reporter gene expression to a subset of adrenocortical cells suggests that 21-OHase/β-gal transgenic mice can be used to draw conclusions regarding cell lineage relationships within the mouse adrenal cortex. We propose that these radial stripes of transgene expression identify cells that have a common clonal origin and, therefore, that the adrenal parenchyma is composed of clonally related cords of cells that extend centripetally from the zona glomerulosa into the inner zonae fasciculata/reticularis. Such an arrangement is entirely consistent with the cellular migration model for growth and maintenance of the adult adrenal cortex, in which cells originating in a multipotent stem cell population are displaced centripetally through the zones, suggesting, in turn, that zone-specific function results from phenotypic modulation of these cells in response to their position within the cortex.

RESULTS

A Steroid 21-OHase Gene Promoter/β-gal Reporter Construct Is Selectively Expressed in Y1 Adrenocortical Tumor Cells

We first investigated the extent to which the 21-OHase gene promoter, when linked to a heterologous reporter gene, is able to recapitulate the adrenal cortex-specific expression pattern of the endogenous 21-OHase gene. To this end, 6.4 kb of the 21-OHase A gene 5′-flanking region were fused, 5 bp downstream of the transcription initiation site, to the *Escherichia coli (E. coli)* lacZ gene (Fig. 1). This gene encodes the *E. coli* glycoside hydrolase, β-D-galactosidase (β-gal) protein, frequently used as a reporter of promoter activity (13). The 6.4-kb 21-OHase gene 5′-flanking region

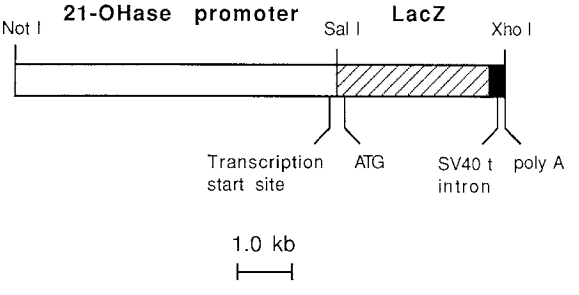

Fig. 1. Structure of 21-OHase Gene Promoter/β-gal Reporter Construct

The 21-OHase gene promoter/β-gal reporter construct was assembled as described in *Materials and Methods* and contains 6.4 kb of 21-OHase gene 5′-flanking sequence linked, 5 bases downstream of the transcription start site, to the complete *E. coli* β-gal coding region, fused at its 3′-end to the SV40 small T-antigen intron and polyadenylation signal. The fragment for microinjection was isolated by double digestion at the unique *Not*I and *Xho*I restriction sites positioned, respectively, at the 5′- and 3′-ends of the promoter-gene fusion.

includes two upstream elements previously shown to be important for expression in transgenic mice (14). The 21-OHase/β-gal reporter construct also contained the SV40 small T-antigen intron and polyadenylation signal, positioned immediately downstream of the β-gal coding region (Fig. 1), to provide splicing and polyadenylation functions required for efficient transgene expression in cell lines and transgenic animals (15).

Substantial β-gal reporter activity resulted from transfection of the 21-OHase gene promoter/β-gal reporter construct into Y1 adrenocortical tumor cells (Fig. 2), which manifest a partially differentiated zona fasciculata phenotype (16). However, this construct failed to yield significant reporter activity when transfected into MA-10 mouse Leydig tumor cells, a steroidogenic testicular tumor cell line that produces progesterone but does not synthesize mineralocorticoids or glucocorticoids (17). Transfection of pSV/β, a reference SV40 promoter-enhancer/β-gal reporter plasmid, into both Y1 and MA-10 cells yielded high levels of β-gal activity in each case. These experiments indicate that the 21-OHase gene 5′-flanking region used in the 21-OHase/β-gal reporter construct contains elements that confer specific expression in Y1 adrenocortical tumor cells but not in MA-10 Leydig tumor cells.

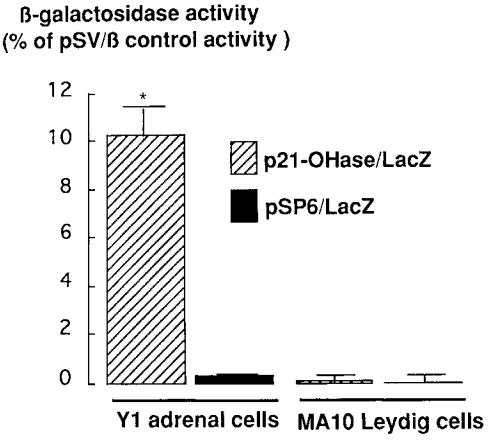

Fig. 2. The 21-OHase Gene Promoter/β-gal Reporter Construct Is Selectively Expressed in Y1 Adrenocortical Tumor Cells

Y1 adrenocortical tumor cells and MA-10 Leydig tumor cells were transiently transfected with 5 μg of the following promoter/reporter constructs: 1) 21-OHase promoter/β-gal, 2) pSP6 promoterless/β-gal control, and 3) pSV/β reference. In each case, 2.5 μg pCAT-control were cotransfected to control for transfection efficiency. Results are presented as a percentage of β-gal activity expressed from the pSV/β vector reference in each cell line (pSV/β reporter activity was 7.0 ± 2.6 U/μg protein in Y1 cells and 121.8 ± 16.5 U/μg protein for MA-10 Leydig cells). Determinations are the mean ± SEM of four (Y1 cells) and three (MA-10 cells) different experiments, each of which consisted of triplicate transfections. Results that show statistical significance at the $P < 0.05$ confidence level (Student's *t* test) are indicated with an *asterisk*.

The 21-OHase Gene 5′-Flanking Region Directs Adrenal Cortex-Specific β-gal Reporter Expression in Transgenic Mice

To ascertain if the 21-OHase gene 5′-flanking region is able to specifically direct heterologous gene expression to the adrenal cortex *in vivo*, the 21-OHase/β-gal reporter construct was introduced into the mouse germ line by pronuclear injection. Histochemical and direct enzyme assays revealed the presence of significant levels of β-gal activity in the adrenal glands of 10 out of 18 founder transgenic animals (18). Sectioning and staining of adrenal glands from G_1 and G_2 offspring of founder 3737 (Fig. 3A) and all other founders showing β-gal reporter activity (not shown) revealed that β-gal staining was confined to the zona glomerulosa and zona fasciculata/reticularis of the adrenal cortex and was undetectable in the adrenal medulla of these animals. The absence of staining from the adrenal cortex of transgene negative littermates (Fig. 3A) and also CBA/Ca and C57Bl/6 progenitor strains (data not shown) indicates that β-gal activity detected in 21-OHase/β-gal mice results from expression of the transgene, rather than endogenous glycoside hydrolase activities. β-gal assay of a panel of tissues from offspring of transgenic mouse (TGM) founder 7911 revealed a 300-fold greater activity in the transgenic adrenal homogenate compared with a negative littermate and also a minor transgenic testicular activity (Table 1), similar to that seen in a minority of 21-OHase/chloramphenicol acetyl transferase (CAT) transgenic founders (14). Further, β-gal activity was only detectable in the adrenal primordium of whole mount-stained TGM 7911 embryos and not in any other tissue (data not shown). Taken together, these data indicate that elements conferring adrenal cortex-specific gene expression *in vivo* are located within 6.4 kb of the 21-OHase gene transcription start site. The only exceptions to the adrenocortical-specific expression were in TGM line 3737, in which staining also was present in the spinal cord, and TGM line 7906 where some peripheral nerves stained blue. The significance of this extraadrenal staining in these lines is not clear, but it is likely to result from integration of the transgene near to, or within, genes normally expressed in the nervous system. Staining is frequently seen in neural tissue of transgenic and knockout mice expressing a β-gal reporter, perhaps because of the abundance of expressed genes in the developing nervous system (19).

Variegated Pattern of β-gal Reporter Staining in the Adrenal Cortex of Adult 21-OHase/β-gal Mice

The adrenocortical specificity of 21-OHase/β-gal transgene expression is consistent with that of the endogenous 21-OHase A gene (12). However, in contrast to the expected pan-cortical pattern of transgene expression, tissue sections of adult adrenal glands from TGM line 3737 21-OHase/β-gal G_1 and G_2 off-

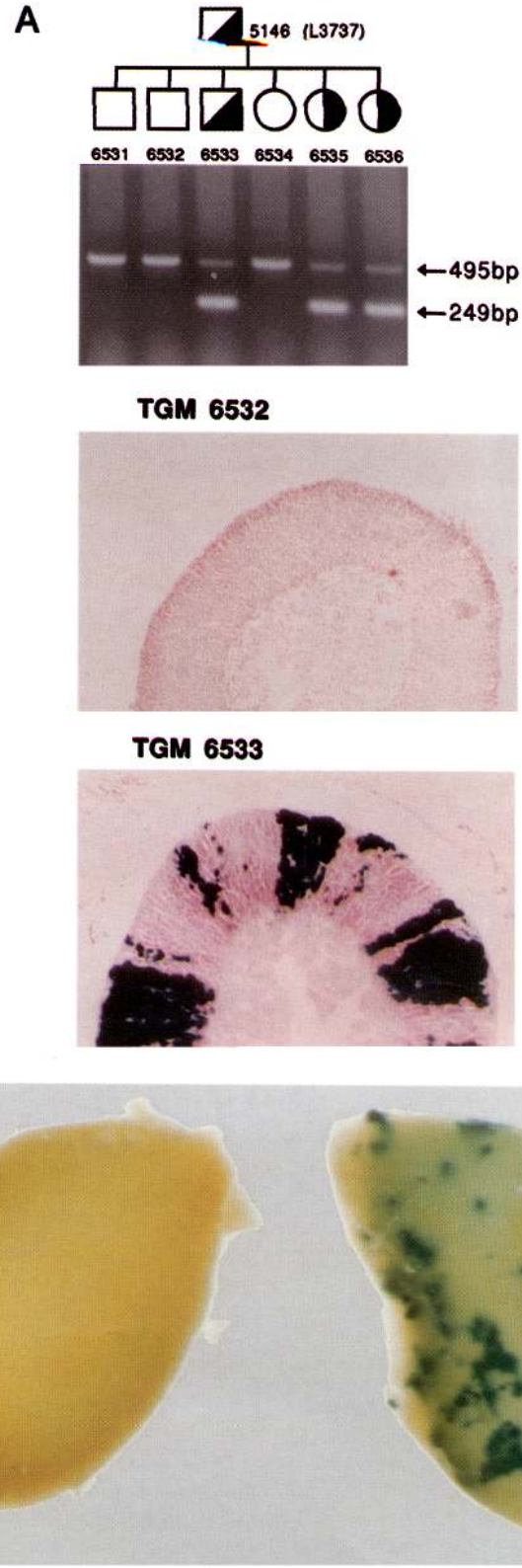

Fig. 3. The 21-OHase/β-gal Transgene Shows a Variegated Pattern of Reporter Expression in the Adrenal Cortex of Transgenic Mice

A, *Upper*, The transgenic status of a heterozygous litter of mice from line 3737, bearing the 21-OHase/β-gal transgene, was determined by PCR-based analysis of tail clip DNA, as described in *Materials and Methods*. Amplification of a 249-bp band indicates the presence of the transgene. *Middle and lower panels*, Equatorial cryostat sections (10 μm) from transgene-positive mice and negative littermates were stained for β-galactosidase activity: *middle*, mouse 6532 (transgene-negative male, 10 months

Table 1. β-gal Reporter Activity in Tissues of TGM Line 7911 21-OHase/β-gal Mice

Tissue	β-gal activity units/mg protein	
	TGM 9653(+)	TGM 9651(−)
Adrenal	30113.6	105.2
Kidney	71.1	62.5
Liver	89.8	60.0
Spleen	62.5	49.7
Heart	21.9	24.5
Lung	27.8	20.7
Brain	17.9	19.2
Testis	337.2	202.2
Muscle	17.5	16.7
Submaxillary	38.2	40.7

A panel of tissues from a 21-OHase/β-gal TGM line 7911 transgenic mouse (mouse 9653; male, 4 months old) and an age- and sex-matched transgenic negative littermate (mouse 9651) were homogenized and assayed for β-gal activity, as described in *Materials and Methods*.

spring exhibited a strikingly variegated pattern of β-gal reporter staining, suggesting that transgene expression occurs only in a limited population of adrenocortical cells (Fig. 3A). Thus, while β-gal reporter activity was confined entirely to the adrenal cortex and was undetectable in the adrenal medulla, it presented in equatorial tissue sections as a pattern of radial stripes of intense staining against a nonstaining background. Significantly, the stripes of staining extend continuously from the glomerulosa, through the fasciculata, to the reticularis, apparently transcending the classic zonal structure of the adrenal cortex. Sections from above or below the equatorial plane, displayed in Fig. 3A, exhibited spots of staining suggestive of columns of reporter-expressing cells transecting the plane of the section at an angle. The stripes of staining seen in two dimensions may therefore result from sectioning through three-dimensional columns of cells, which radiate in the plane of the section, while the few isolated areas of staining seen result from sectioning through columns radiating above or below the plane of the section. Staining of whole adult adrenal glands reveals discrete areas of staining on the surface of the gland, also suggestive of reporter-expressing cells radially disposed in three dimensions within the adrenal cortex (Fig. 3B). An essentially identical pattern of variegated adrenocortical β-gal staining was seen in frozen tissue sections prepared from TGM line 3737 mice ranging from 6 weeks to 1 yr of age, the pattern of staining being similar in males and females, with the exception that the proportion of staining cells in females was reduced by about one third (not shown). Since adult female mouse adrenal glands are roughly 50% larger than their male counterparts, due to the vacuolated nature of the tissue and the presence, under certain physiological conditions, of an additional sex hormone-responsive X-zone between the zona fasciculata and reticularis, it is probable that approximately the same number of adrenocortical cells express the transgene in both male and female animals. Male and female G_1 and G_2 offspring of the other nine expressing TGM 21-OHase/β-gal lines all showed a similar pattern of adrenal cortex-specific variegated staining, although the proportion of staining cells differed between lines. This ranged from widespread, strong staining throughout the adrenal cortex (one line: TGM line 7911), through a localized strong staining (seven lines), to localized weak staining (two lines). Of the lines studied in detail, TGM line 3737 and 7906 mice exemplified the localized strong staining pattern, while TGM line 7911 offspring showed the highest density of adrenal β-gal staining among all the lines tested, amounting to 80–90% of adrenocortical cells. Although the proportion of cortical cells expressing the reporter transgene may be influenced by the site of transgene integration, the variegated pattern of expression cannot be due to transgene mosaicism because G_1 and G_2 animals were examined for each of the 10 independent expressing lines. Thus, the variegated reporter expression must be a property of 21-OHase/β-gal transgene expression in the mouse adrenal environment.

21-OHase/β-gal Transgene mRNA Shows a Variegated Distribution in the Adrenal Cortex of Transgenic Mice

In situ hybridization of serial paraffin sections of transgenic adrenal glands from mice of TGM line 3737, with a β-gal coding region probe, shows a variegated distribution of 21-OHase/β-gal transgene mRNA in the adrenal cortex, similar to that seen for the β-gal reporter (Fig. 4A). In contrast, *in situ* hybridization of serial sections with a 21-OHase probe shows that the endogenous mouse 21-OHase gene mRNA is expressed throughout the adult adrenal cortex (Fig. 4B), consistent with the known expression pattern for this gene (12). Significantly, the distribution of endogenous 21-OHase mRNA appears to be unaffected by expression of the transgene. Colocalization of 21-OHase/β-gal transgene mRNA with the active β-gal reporter protein was confirmed by subjecting serial tissue sections from adrenals of TGM line 3737 transgenic mice to either *in situ* hybridization with an antisense cRNA probe to the β-gal coding region or immunostaining with a β-gal-specific antibody. Staining patterns ob-

old); *lower*, mouse 6533 (transgene-positive male, 10 months old). B, Adrenal glands dissected from line 3737 transgene-positive and transgene-negative mice were fixed in 0.2% (vol/vol) glutaraldehyde and stained for 16 h in X-gal solution to reveal β-gal activity. *Right*, Transgene-positive adrenal gland (TGM 6533; male 10 months old), *Left*, Age- and sex-matched transgene-negative adrenal gland; magnification ×140.

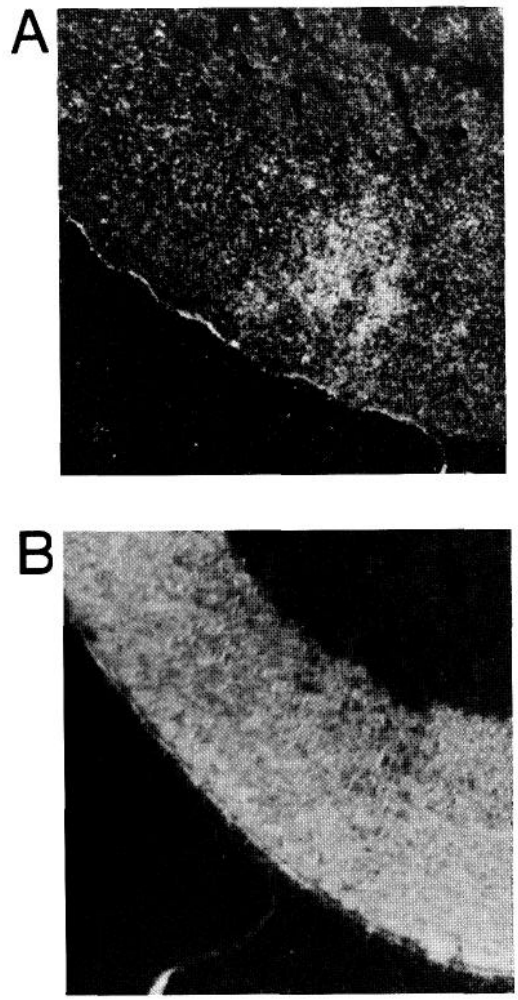

Fig. 4. *In Situ* Hybridization Reveals a Variegated Pattern of 21-OHase/β-gal Transgene mRNA in the Adrenal Cortex of Transgenic Mice

In situ hybridization of serial paraffin sections (7 μm) from the adrenal gland of a transgene-positive mouse from founder line 3737 (mouse 8048 male, 4 months old), was carried out using either (A) a β-gal cRNA probe or (B) a 21-OHase cRNA probe. Complementary RNA probes were generated and sections hybridized as described in *Materials and Methods*.

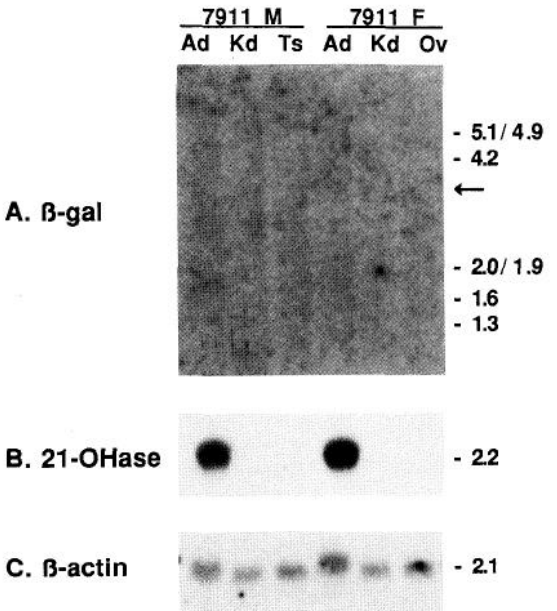

Fig. 5. 21-OHase/β-gal Transgene mRNA Is Present at Lower Levels than mRNA for the Endogenous 21-OHase Gene in the Adrenal Cortex of Transgenic Mice

Northern blot analysis of 10 μg poly A+ RNA isolated, respectively, from pooled adrenal glands from four littermates: adrenal (Ad), kidney (Kd), and testis (Ts) or ovary (Ov) of 3- to 4-month-old male or female TGM 7911 transgene-positive mice. The blot was hybridized successively with (A) a full length *E. coli* β-gal coding region cDNA probe; (B) a 495-bp 21-OHase gene probe generated by PCR as described in *Production and Analysis of Transgenic Mice*; and (C) a full length rat β-actin cDNA probe, and autoradiographed, respectively, for 10 days, 4 h, and 18 h. 7911 M and 7911F indicate RNAs prepared from, respectively, male and female animals. Size markers are indicated in kilobases. The anticipated position of a correctly spliced β-gal reporter mRNA is shown by an *arrow*.

tained by both methods in neighboring sections were essentially identical (data not shown), indicating that the variegated pattern of β-gal reporter protein activity reflects a variegated distribution of transgene mRNA.

Messenger RNA for the 21-OHase/β-gal Transgene Is Present at Much Lower Levels than mRNA for the Endogenous 21-OHase Gene

Unexpectedly, 21-OHase/β-gal transgene mRNA was undetectable after 10 days of autoradiographic exposure in Northern blot analysis of pooled adrenal poly A+ RNA from TGM line 7911 (Fig. 5), in which the highest proportion of β-gal-expressing adrenocortical cells was observed. In contrast, hybridization with a 21-OHase gene probe revealed a mRNA of approximately 2.2 kb in adrenal RNA, corresponding to the size of the endogenous mouse 21-OHase mRNA, while mRNA for β-actin was detectable in all samples analyzed. Qualitatively similar data were obtained for lines 3737 and 7906 using total adrenal RNA, and inclusion of suitable controls showed that the β-gal-coding region cDNA probe was able to efficiently detect a β-gal target sequence within total RNA (data not shown). Given that the 21-OHase/β-gal transgene is expressed in only a proportion of adrenocortical cells, one would expect the intensity of transgene mRNA signal to be reduced by the ratio of transgene-expressing to nonexpressing cells, if the transgene and endogenous 21-OHase gene are being expressed at similar levels. The absence of a transgene mRNA signal on Northern blots suggests that, in addition to transgene expression occurring only in a subset of adrenocortical cells, either the transgene is transcribed at much lower levels than the endogenous 21-OHase gene in these lines and/or transgene mRNA has a shorter half-life than its endogenous counter-

part. Experiments aimed at more sensitive detection of 21-OHase/β-gal mRNA and to distinguish between these possibilities are underway.

The 21-OHase/β-gal Transgene and the Endogenous 21-OHase Gene Are Activated at Similar Developmental Stages

Since the fusion of a gene promoter to a heterologous reporter gene can sometimes result in the reporter moiety acting in *cis* to delay the onset of expression (20), it is important to determine whether 21-OHase/β-gal transgene expression is correctly regulated during development. X-gal staining of partially dissected line 7911 transgenic embryos revealed β-gal reporter activity in the adrenal primordia at embryonic day 11.5 and 12.5 (E11.5 and E12.5) of gestation (Fig. 6A). This appeared as a diffuse blue staining in the region medial to the urogenital ridge (Fig. 6A) and was completely absent from transgenic negative littermates (not shown). At E15.5 and E17.5 after the adrenal gland has become a morphologically distinct structure, staining is more intense, although variegated, covering 80–90% of the visible surface of the gland, consistent with the degree of staining seen in adult adrenal glands of this line. By comparison, *in situ* hybridization of paraffin sections of nontransgenic mouse embryos with a 21-OHase cRNA probe showed that expression of the endogenous 21-OHase gene could also be detected in the E11.5 adrenal primordium (Fig. 6B). At E12.5 the 21-OHase signal is stronger as the adrenal primordium enlarges. At E14.5 and E17.5, endogenous 21-OHase gene expression is further enhanced compared with earlier stages and, particularly at E17.5, has adopted the classical adrenal pan-cortical distribution with some islands of expression remaining in the adrenal medulla, presumably as a result of an as yet incomplete resolution of cortex and medulla. These results indicate that both the 21-OHase/β-gal transgene and the endogenous 21-OHase gene are activated at similar developmental stages. This, in turn, suggests that the 6.4-kb 21-OHase gene 5′-flanking region used in the transgene contains all the developmental cues required for the appropriate onset of expression during development.

Variegated Pattern of β-gal Reporter Staining in Developing Adrenal Glands of 21-OHase/β-gal Embryos

Transgenic embryos of line 7911 were chosen for analysis of 21-OHase/β-gal transgene ontogeny by virtue of displaying the highest proportion of adrenocortical β-gal reporter staining. Onset of 21-OHase/β-gal transgene expression was also noted in adrenal primordia of partially dissected E11.5-E12.5 embryos from lines 3737 and 7906 (data not shown). In keeping with the expression patterns seen in adult embryos of these lines, a much smaller proportion of the adrenal primordium was stained for β-gal activity, making detection difficult in frozen sections of embryos from these time points. However, the lower percentage of β-gal-positive cells in these lines should, in principle, provide a clearer picture of the radial patterning at later embryonic stages as 21-OHase/β-gal transgene expression is up-regulated. Indeed, β-gal reporter activity was clearly detectable in frozen sections of E15.5 and E16.5 adrenals of line 3737 (Fig. 7), presenting as a variegated pattern of staining in a proportion of cells of the developing adrenal gland, as was the case for adult adrenal glands of this line (Fig. 3). Since the adrenal cortex and adrenal medulla are incompletely resolved at these time points, it is unclear to what extent the variegated expression pattern results from intermingling of adrenal cortex and medulla. However, the small overall proportion of staining cells argues that a significant number of adrenocortical cells are not expressing the transgene at these embryonic stages. Significantly, while E15.5 and 16.5 embryos show a variegated pattern of transgene expression, the embryonic staining pattern is distinguished from that in the adult by the absence of fully developed radial stripes of cortical staining. Instead, even in equatorial sections of the embryonic adrenal gland, it presents as islands of staining cells in a nonstaining background, with a suggestion at the later stage that some of the islands may be elongating to form primitive stripes (Fig. 7, *arrows*). This different patterning presumably reflects the fact that the classic zonation of the cortex is not completely established at these embryonic stages. Experiments are presently underway to discover at what point in development the radial striped pattern, characteristic of the adult adrenal cortex, becomes fully established.

DISCUSSION

The results presented here show that 6.4 kb of the mouse steroid 21-OHase A gene 5′-flanking region are sufficient to direct specific LacZ reporter gene expression, both in Y1 adrenocortical tumor cell cultures and the adrenal cortex of transgenic mice. The 6.4-kb 21-OHase gene 5′-flanking region used in the 21-OHase/β-gal transgene contains regulatory elements, located 5–6 kb upstream of the transcription start site, that have previously been shown to be important for expression of a CAT reporter gene in the adrenal gland of transgenic mice (14). However, these authors did not examine the localization of 21-OHase/CAT expression within the adrenal gland, although two animals were shown to posses an additional minor gonadal CAT activity. The current study advances these observations in three important respects. First, the specific nature of 21-OHase/β-gal transgene expression in Y1 adrenocortical tumor cells is confirmed by the fact that no reporter activity was seen in MA-10 mouse Leydig tumor cells, a steroidogenic testicular tumor cell line that produces progesterone but does not syn-

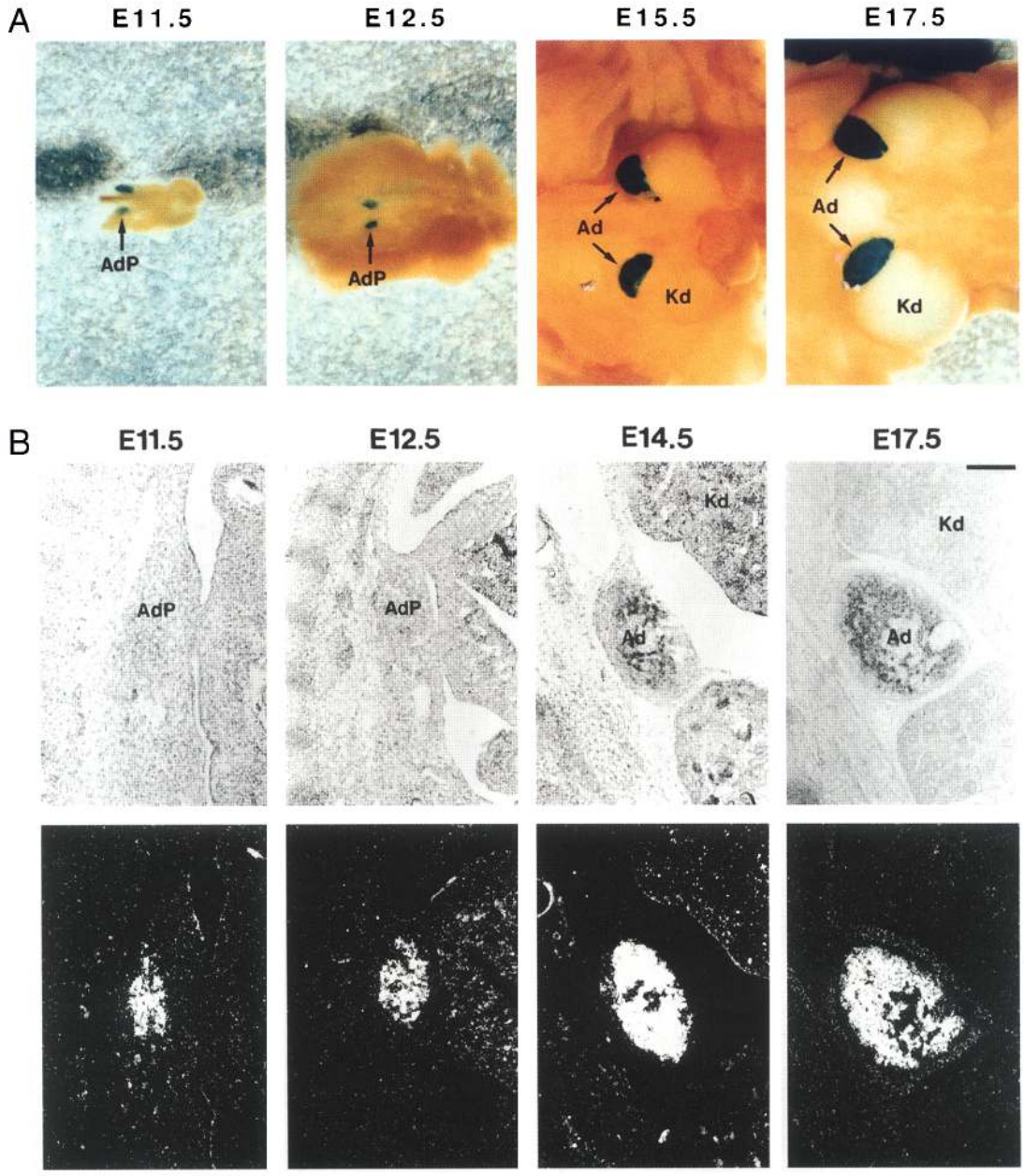

Fig. 6. Ontogeny of the 21-OHase/β-gal Transgene Mirrors that of Endogenous 21-OHase Gene Expression in the Mouse Adrenal Cortex

21-OHase/β-gal transgenic-positive embryos and nontransgenic controls were obtained from timed pregnant mice at the indicated time points and subjected either to partial dissection and histochemical staining for β-gal reporter activity or sectioning and *in situ* hybridization with a 21-OHase cRNA probe. A, Histochemical staining of TGM founder line 7911 transgenic embryos for β-gal reporter activity; age of the embryo is indicated in days post coitus. B, *In situ* hybridization of nontransgenic mouse embryos: *upper panel*, brightfield image of 21-OHase *in situ*; *lower panel*, darkfield image of 21-OHase *in situ*. AdP, Adrenal primordium; Ad, adrenal gland; Kd, kidney. Dissected embryos are ×57.5 magnification, and tissue sections are ×115 magnification.

thesize mineralocorticoids or glucocorticoids (17). Second, we show that the 21-OHase/β-gal transgene is specifically expressed in both the zona glomerulosa and the zona fasciculata/reticularis of the adrenal cortex but not in the adrenal medulla of transgenic mice. Finally, transgene expression is appropriately regulated during mouse embryonic development, β-gal reporter expression first being detected at E11.5 in the adrenal primordium coincident with the onset of endogenous 21-OHase gene expression. Taken together, these results suggest that elements conferring adrenal cortex-specific and developmentally appropriate gene expression *in vivo* are located within the first 6.4 kb of 21-OHase

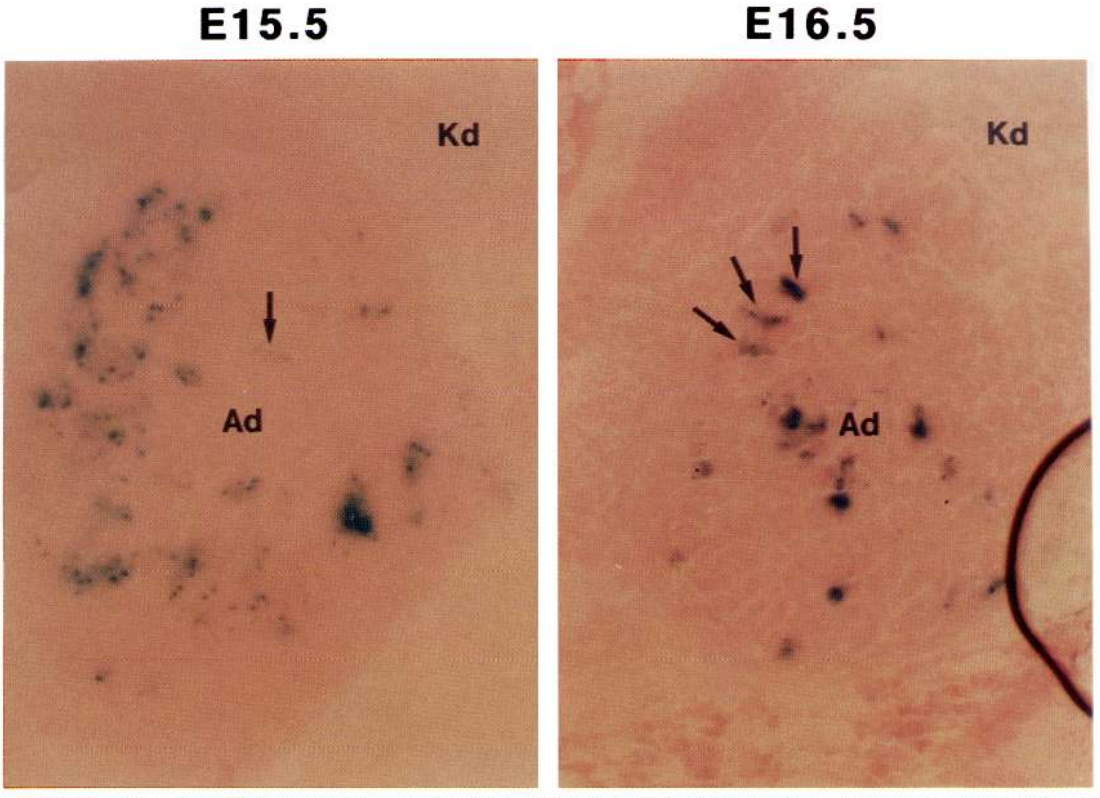

Fig. 7. Embryonic 21-OHase/β-gal Transgene Expression Is Revealed as Variegated Islands of β-gal Reporter Staining in the Developing Adrenal Gland

TGM line 3737 21-OHase/β-gal transgenic-positive mouse embryos were obtained from timed pregnant mice at the indicated time points and were cryosectioned transversely (10 μm), fixed in 0.2% (vol/vol) glutaraldehyde, and histochemically stained for β-gal reporter activity; the age of embryos is indicated in days post coitus. Ad, Adrenal gland; Kd, kidney. Magnification ×140.

gene 5′-flanking region upstream of the transcription start site.

Although 21-OHase/β-gal transgene expression is highly adrenocortical-specific, the β-gal reporter displays a strikingly variegated pattern of radial staining in the adrenal cortex of all 10 independent transgene-expressing mouse lines examined. *In situ* hybridization analysis reveals that 21-OHase/β-gal transgene mRNA displays a similar variegated adrenocortical distribution to the β-gal reporter protein. Together these data suggest that 21-OHase/β-gal transgene expression occurs only in a limited population of cells of the adrenal cortex, while it is transcriptionally silent in others. This contrasts sharply with the endogenous mouse 21-OHase gene, which is expressed throughout the adrenal cortex (12). The nature of the mechanism giving rise to the variegated pattern of 21-OHase/β-gal transgene expression remains unclear. However, it must be a property of 21-OHase/β-gal transgene expression in the mouse adrenal environment, rather than the transgene insertion site, since variegated expression was seen in all 10 independent 21-OHase/β-gal transgene-expressing mouse lines examined. A variety of effects of the β-gal reporter on heterologous promoter activity in transgenes have been noted, including suppression of normal levels of activity and down-regulation of an adjacent promoter in a mixed transgene array (20, 21). In the case of the 21-OHase/β-gal transgene, juxtaposition of the β-gal-SV40/small T-antigen intron moiety downstream of the 21-OHase promoter may also lead to the epigenetic silencing of promoter activity in a proportion of cortical cells by, for example, methylation (22). This might arise as a consequence of the prokaryotic origin of the reporter moiety, its high frequency of CpG dinucleotides causing it to be marked out as foreign during its passage through germ line or preblastula stages (20). The distinctive variegated pattern of β-gal staining seen in 21-OHase/β-gal transgenic adrenals could result from the escape of a proportion of cell sublineages from silencing of the β-gal reporter gene in, for example, an early population of stem cells. Methylation-dependent variable inheritance of expression patterns, showing that methylation differences can exist between cells within a tissue, has already been demonstrated for a *hsp68/LacZ* transgene in the mouse (23). Proliferation of stem cells followed by centripetal displacement could then give rise to radially distributed strings of clonal cells that either express or do not express the transgene.

Surprisingly, in the three transgenic lines examined in detail, 21-OHase/β-gal transgene steady state mRNA levels detected *in vivo* are significantly reduced relative to those of the endogenous 21-OHase gene.

This cannot be fully explained by variegated expression of the 21-OHase/β-gal transgene and suggests that a distinct mechanism may be affecting transgene mRNA steady state levels in cells able to express the transgene. Thus, in Northern analysis of poly A+ adrenal RNA from line 7911 transgenic mice, in which 80–90% of the adrenal cortex stains for reporter expression, transgene mRNA cannot be detected even after long Northern exposure, although endogenous 21-OHase mRNA gives a strong signal (Fig. 5). Thus, in cells expressing the 21-OHase/β-gal transgene, either the transgene is transcribed at much lower levels than the endogenous 21-OHase gene or transgene mRNA has a shorter half-life than its endogenous counterpart. Little is known about the stability of LacZ reporter gene mRNA in mammalian systems, although this is likely to depend on the transgene construct and, in particular, the polyadenylation signal being used. It seems unlikely that additional elements should be required for maximal 21-OHase promoter activity, since high levels of transgene expression, comparable to the expression of the endogenous 21-OHase gene, are seen in transgenic rats in which the 6.4-kb 21-OHase gene 5′- flanking region is linked to a temperature-sensitive SV40 T-antigen reporter gene (I. Viard, S. D. Morley, Y. Ikeda, K. L. Parker, and J. J. Mullins, in preparation). It is probable therefore that the β-gal-SV40/small T-intron reporter moiety acts in *cis* to down-regulate 21-OHase promoter activity in cells that express the transgene. This could occur either because the β-gal reporter moiety acts directly in *cis* to down-regulate the 21-OHase gene promoter or by a deleterious effect of the SV40 small T-antigen intron, causing, for example, enhanced use of cryptic splice sites resulting in significant levels of translationally inactive mRNAs (24, 25).

Transgene expression in only a proportion of expression-competent cells in a tissue, resulting in a variegated phenotype, is by no means unprecedented, since several other transgenes, and also an endogenous mouse ecotropic virus locus, display correct tissue-specific expression but also exhibit variegated expression when maintained in a hemizygous state (23, 26–28). Methylation and other epigenetic changes influencing gene expression usually occur in progenitor cells early in development and are then stably propagated in all daughter cells that give rise to somatic tissues (23). This implies that early modification of the 21-OHase/β-gal transgene in a proportion of adrenocortical progenitors could give rise to two expression phenotypes (on or off), resulting in two independent clonally derived somatic cell lineage classes in the adrenal cortex, one of which will express the β-gal reporter and one of which will not. The situation where only a proportion of cells in a tissue possess an active transgene can also be created artificially by injecting LacZ-expressing retroviruses into early mouse embryos (29) or by creating chimeric rats by amalgamating preimplantation embryos of two congenic strains that express different markers (30). In all three cases, the presence of genetically or epigenetically distinct progenitor cells should result in distinct somatic cellular lineages, only some of which express the reporter moiety, allowing their fate to be monitored against the background of nonexpressing cells. Such lineage-tracing techniques have allowed the origins of certain subsets of neurons to be determined (20, 29) and have enabled the conclusion that in several organs, including the liver, cells expand clonally to yield randomly distributed patches but, in general, do not migrate (30). The present results suggest a different origin for clonally related cells in the adrenal cortex. Crucially, the radial pattern of β-gal reporter staining, arising from 21-OHase/β-gal transgene expression, extends continuously from the zona glomerulosa, through the zona fasciculata, to the zona reticularis/medulla boundary, transcending the classic concentric zonal arrangement, but following the morphological arrangement of cells and blood supply of the adult adrenal cortex. The existence of such a radial striped pattern of 21-OHase/β-gal transgene expression suggests that the adrenal parenchyma is composed of clonally related cords of cells that extend centripetally from the zona glomerulosa into the inner zonae fasciculata/reticularis. This implies that cells of the different zones have a common origin and that migration between the zones, accompanied by phenotypic modification in response to their immediate environment, is the normal mechanism of renewal in the adult unstressed adrenal gland. The radial striped pattern of 21-OHase/β-gal reporter expression is thus entirely consistent with the classic cellular migration hypothesis for the maintenance of the adult adrenal cortex, in which cells that arise in the zona glomerulosa migrate centripetally through the zona fasciculata to the zona reticularis and cannot be reconciled with autonomously replicating zonal cell populations, as proposed by the zonal theory. A modified version of the cellular migration hypothesis, the proliferative intermediate zone hypothesis (31), suggests that a single pool of precursor cells, located between the zona glomerulosa and zona fasciculata, gives rise to cells that express either the zona glomerulosa or zona fasciculata phenotype. If this model is correct, the extension of the variegated pattern across zonal boundaries predicts that these precursor cells must have already undergone the epigenetic event that silences transgene expression. Further studies are required to distinguish between these possibilities.

The current data extend conclusions on the outside to inside direction of cell division during the rapid growth phase of adrenocortical regeneration after enucleation of the gland (2, 11), by showing that a similar organization may play a role in maintenance of the unstressed gland. Since the mouse adrenal gland is essentially a flattened sphere, it is difficult to draw any direct conclusions about the direction of cellular migration from the current results. However, the observed constant thickness of most of the β-gal radial stripes in equatorial sections of the 21-OHase/β-gal

transgenic adrenal cortex and the known predominance of mitoses in the zona glomerulosa and cell death in the zona reticularis (3, 4, 8–10) strongly suggest that migration is centripetal rather than centrifugal. Work in chimeric rats, expressing different alloantigens of the major histocompatibility system which can be detected in sections of chimeric adrenal glands with specific antibodies, also revealed the presence of parallel cords of cells throughout all three zones of the adrenal cortex (30). However, the pyramidoid (pyramid with curved corners) shape of the rat adrenal gland, coupled with the constant thickness of the columns of cells, implies that cellular migration in the adrenal gland must be centripetal, since otherwise columns of cells migrating outward would show a thickening or wedge shape at the corners to establish the extra tissue needed for the outer circumference of the cortex (32). The variegated expression of the 21-OHase/β-gal transgene enhances and generalizes these conclusions by demonstrating radial organization of adrenocortical cell lineages in a second species, the mouse. More importantly, the radial organization and presumed centripetal migration of adrenal cells in this study occur in a nonchimeric animal, such that all cells have an identical genetic composition and presumably are distinguished only by the epigenetic modification that leads to variegated expression of the transgene.

In addition to allowing us to analyze the pattern of adrenocortical organization in nonchimeric animals, our studies also provide novel insights into the development of this variegation and the presumed role of cellular migration during embryological development of the adrenal gland. The adrenal cortex is first morphologically distinguishable on mouse E12.5–13 as a condensation of mesoblast in the coelomic mesoderm, medial to the urogenital ridge (33), although Steroidogenic Factor-1 (34) and 21-OHase activities (this paper and Ref. 12) are detectable earlier, and a glucocorticoid synthetic capacity, including a 11β-hydroxylase activity, is probably already present (35, 36). Over the next 2–3 days, the gland enlarges by recruitment of coelomic epithelium from the region of the gastric mesentery and also of cells arising from clusters of sympathetic chromaffin cells, which will form the adrenal medulla. At this point, adrenal cortex and medulla components are intermingled. The classic resolution of a concentric cortex and medulla and subsequently of the individual zones of the cortex only occurs over E17.5–19.5, shortly before birth. This includes the appearance of a morphological zona glomerulosa and aldosterone synthetase activity, which enables the commencement of mineralocorticoid synthesis. Thus, the adrenal cortex increases in size initially by recruitment of cells from outside the organ, and probably only later does cell division within the organ become significant. At birth, the adrenal gland undergoes a significant regression accompanied by reduction in weight, followed by a rapid growth phase to approach the weight of the mature organ. All of these considerations suggest that processes governing adrenocortical development are likely to be distinct from maintenance and renewal of the adult organ. Equatorial frozen sections of E15.5 and E16.5 embryos of line 3737 revealed a variegated pattern of staining in the adrenal cortex, as was the case for adult adrenal glands. However, the embryonic staining pattern presents as islands of staining cells in a nonstaining background, rather than the radial stripes of staining seen in the adult adrenal cortex, with a suggestion at the later stage that some of the islands may be elongating to form primitive stripes. This suggests that centripetal migration may not be the major mechanism of cell movement within the E15.5–16.5 adrenal cortex, which may reflect the fact that the classic zonation of the adrenal cortex has yet to be established at these stages. Writhing of cell columns in and out of the plane of the section, due to mixing of cortical and medullar components, cannot be excluded. However, the embryonic patterning of 21-OHase/β-gal expression could indicate that adrenocortical cells throughout the embryonic organ have the ability to replicate and that progenitor cells and mitosis only become localized at the periphery of the gland as the zonal structure becomes established. This is consistent with the idea that the development of a zonal structure is potentiated by the establishment of a glucocorticoid gradient within the adrenal cortex dictating both adrenocortical phenotype and replicative potential in response to position (2). This implies that confinement of replicating cells to the peripheral zona glomerulosa will occur late in embryonic development, with this process being incomplete in E15.5–16.5 mouse adrenals. Thus, centripetal migration of adrenocortical cells may only be established at the end of embryogenesis or in early postnatal life. Accordingly, experiments are presently underway to discover at what point in adrenocortical development the radial striped pattern characteristic of the adult 21-OHase/β-gal adrenal cortex becomes fully established.

In conclusion, the radially variegated expression of the 21-OHase/β-gal transgene expression seen here suggests that the adrenal parenchyma is composed of clonally related cords of cells that extend centripetally from the zona glomerulosa into the inner zonae fasciculata/reticularis; this finding is entirely consistent with a centripetal migration of cells between the cortical zones to maintain the adult mouse adrenal cortex. Our results further suggest that distinct cellular mechanisms contribute to adrenal development at earlier stages of embryogenesis, before centripetal migration is established. Transgenic mice displaying a variegated pattern of 21-OHase/β-gal transgene expression, specifically in the adrenal cortex, have fortuitously provided us with a marker of adrenocortical cell lineage. The availability of these mice should facilitate further experiments designed to monitor and dissect the mechanism of adrenocortical cell migration during development and maintenance of the adult organ.

MATERIALS AND METHODS

Materials

Restriction and modification enzymes were purchased from Boehringer-Mannheim (Lewes, UK; or Indianapolis, IN). Reagents for cell culture were from GIBCO BRL (Paisley, Scotland). Radionucleotides were obtained from Amersham International (Buckinghamshire, UK) or New England Nuclear-E.I. Dupont (Boston, MA). Oligonucleotides were synthesized by Oswell DNA Services (Edinburgh, UK) using an Applied Biosystems 380B synthesizer. Deoxynucleoside triphosphates and random hexamers were ordered from Pharmacia (Milton Keynes, UK). Bradford protein determination reagent (37) was produced by Bio-Rad (Hemel Hempsted, UK). 4-Methylumbelliferone, 4-methylumbelliferyl β-D-galactoside and TESPA (3-aminopropyl-triethoxysilane) slide-coating solution were from the Sigma Chemical Co. Ltd. (Poole, Dorset, UK). Tissue-Tek OTC compound embedding medium was manufactured by Miles Inc. (Elkhart IN). Histo-Clear histological clearing reagent was from National Diagnostics (Atlanta, GA). *In situ* hybridization was performed using reagents purchased from Novagen, Inc. (Madison, WI).

21-OHase Promoter/β-gal Reporter Construct

All molecular manipulations were carried out by standard techniques (38). The 21-OHase promoter/β-gal reporter construct contained 6.4 kb of gene 5′-flanking region previously isolated from murine BALB/c cosmid genomic libraries (39). The promoter fragment was introduced into the plasmid pSP64/β-gal/small-t via an artificial *Xho*I restriction site positioned 5 bases downstream from the promoter transcription start site. pSP64/β-gal/small-t carries the complete β-gal coding region fused at its 3′-end to the SV40 small T-antigen intron and polyadenylation signal. To facilitate molecular manipulations, the original polylinker in pSP64/β-gal/small-t was first replaced with one containing 5′-*Not*I, *Eco*RI, *Sal*I, *Pst*I, *Bgl*II, and *Xba*I-3′ sites. The 21-OHase promoter fragment was cloned into the *Not*I and *Sal*I sites such that a *Not*I site was retained at the 5′-end of the promoter, but the artificially introduced *Xho*I site was destroyed by the *Xho*I/*Sal*I fusion.

Cell Culture and Transfection

Y1 adrenocortical tumor cells (16) were grown in DMEM/F12 (1:1) medium containing 15 mM-HEPES, supplemented with 15 mM-sodium bicarbonate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% (vol/vol) FCS, at 37 C in a humidified atmosphere of 5% CO_2. MA-10 mouse Leydig tumor cells (17) were cultured, under similar environmental conditions, in RPMI 1640 medium supplemented with 15% (vol/vol) horse serum. In each case 2×10^5 cells per 60-mm dish were transfected with the 21-OHase promoter/β-gal reporter construct (5 μg/dish), using the calcium phosphate method (40). pSV/β (5 μg/dish) (Clontech), a reference SV40 promoter-enhancer/β-gal reporter plasmid, was transfected in parallel with the 21-OHase promoter/β-gal reporter constructs as a reference for β-gal expression. pCAT-control (2.5 μg/dish) (Promega), which contains a CAT reporter gene driven by the SV40 promoter and enhancer sequences, was cotransfected to control for transfection efficiency, data being corrected appropriately. After cell lysis, cellular protein was determined by the Bradford (37) method. CAT activity was determined by production of [^{3}H]acetyl chloramphenicol by cell lysates (41).

β-gal Assays in Cell and Tissue Extracts

Cell and tissue lysates were assayed for β-gal activity by the methylumbelliferone fluorescence method (42). Briefly, cells were washed off plates directly with 1 × reporter lysis buffer (Promega No. E3971), while tissues were coarsely minced and then homogenized in the same buffer. Lysates and tissue homogenates were centrifuged at 12,000 × *g* for 5 min in an Eppendorf centrifuge and clear supernatants were stored at −70 C or used immediately for enzyme assay. β-gal activity units were arbitrarily defined as the enzyme activity resulting in the conversion of 0.1 nmol 4-methylumbelliferyl β-D-galactoside to 4-methylumbelliferone in 30 min at 37 C. A standard curve was prepared from purified *E. coli* β-gal (Boehringer Mannheim No. 567799) over the concentration range 0–1.28×10^{-4} U/reaction tube (0–200 pg protein). In this assay, one 4-methylumbelliferone unit was equivalent to 5.12×10^{-5} *E. coli* β-gal activity units. Results of enzyme assays from cell culture lysates are the mean ± SEM of a minimum of three different experiments each of which consisted of triplicate transfections. Statistical significance was determined by Student's *t* test at the $P < 0.05$ confidence level.

Production and Analysis of Transgenic Mice

All animal work was carried out in accord with the highest standards of humane animal care as detailed in the Animals (Scientific Procedures) Act (UK) 1986 and in the NIH Guidelines for the Care and Use of Laboratory Animals. The DNA fragment for microinjection was excised from the 21-OHase promoter/β-gal reporter construct by double digestion at polylinker *Not*I and *Xho*I restriction sites, followed by preparative agarose gel electrophoresis and cesium gradient centrifugation to remove contaminating vector sequences (43). Transgenic mice were prepared by established methods (43), using F1 hybrid (CBA/Ca × C57Bl/6) mice as donors and recipients. Positive founder animals were identified by Southern blotting and/or PCR-based analysis of tail clip DNA and back-crossed to CBA/Ca animals to establish transgenic lines. PCR analysis: Triplex PCR reactions contained 1 μM each of a forward primer, which recognizes both the transgene and the endogenous 21-OHase gene promoter (5′-CAGGAAGGGACCTGAAGC-3′) and two reverse primers specific either for the *E. coli* β-gal portion of the transgene (5′-CCATTCAGGCTGCGCAAC-3′) or the endogenous 21-OHase A gene coding region (5′-GGAAACTGAGCAAGGGTC-3′). Reactions (25 μl) in 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, contained 0.2 mM-each dATP, dCTP, dGTP, dTTP, 0.25 U *Taq* DNA polymerase (Promega), and 0.5 μg genomic DNA. These were overlaid with 25 μl mineral oil (Sigma No. M-3516) and denatured initially at 94 C for 5 min, followed by 30 cycles of 94 C/1 min, 62 C/1 min, and 72 C/1.5 min, and finally by one cycle of 72 C/10 min and 25 C/1 min. Products of the PCR reaction were analyzed on a 0.8% (wt/vol) agarose gel. Amplification of 21-OHase/β-gal transgenic positive mouse DNA yielded a diagnostic fragment of 249 bp, while DNA from all mice yielded a control band of 495 bp corresponding to the endogenous 21-OHase A gene.

Preparation of RNA

Total RNA was purified from pools of six to eight adrenal glands from mature male or female mice using RNAzol reagent (44). Poly A+ mRNA was isolated using Oligo dT affinity resin (Boehringer-Mannheim), according to the manufacturer's instructions.

Northern and Southern Blot Analysis

Poly A+ RNA (10 μg) and size markers (5 μg total adrenal RNA and 5 μg *Eco*RI/*Hin*dIII digest of λ-DNA) were denatured with glyoxal (45), resolved on a 1.2% (wt/vol) agarose gel containing 10 mM sodium phosphate, pH 7.0 buffer, and capillary transferred to positively charged nylon membranes (Boehringer 1417240) in 20 × saline sodium citrate (SSC) (1

× SSC = 0.15 M NaCl-0.015 M sodium citrate, pH 7.0). The RNA was deglyoxylated by baking blots at 80 C for 30 min and immobilized by irradiation with 0.24 J UV light in a Stratagene 1800 UV cross-linker. Size markers were cut off the blot and visualized by staining for 5 min in 0.04% (wt/vol) methylene blue in 0.5 M NaOAc, pH 5.5, followed by destaining for 30 min in distilled water. Similarly, 10 μg genomic DNA prepared from tail biopsies (38) were digested to completion with various restriction enzymes, fractionated on a 0.8% (wt/vol) agarose gel, and transferred and immobilized as above. Blots were hybridized under standard conditions (38) with ^{32}P-labeled DNA probes prepared by a modification (46) of the random priming method (47) and washed finally with either 0.5 × or 0.2 × SSC at 68 C.

Adrenal Sectioning and β-gal Histochemistry

Animals were killed by vertebral dislocation after carbon dioxide anesthesia. Tissue fragments were immediately removed by dissection, rinsed briefly in PBS, and either fixed and stained directly as described below or mounted in OTC compound embedding medium, followed by rapid freezing on dry ice. OTC blocks were stored at −70 C until use. In some cases, to expedite analysis or when the founder failed to transmit the transgene, animals were subjected to unilateral adrenalectomy under halothane anesthesia. Frozen sections (10 μm) mounted on TESPA-treated microscope slides were fixed for 5 min in 100 mM Na phosphate, pH 7.3, 0.2% (wt/vol) glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$, washed three times for 5 min in 100 mM Na phosphate, pH 7.3, 2 mM $MgCl_2$, and stained for 12–16 h, at 37 C, in X-gal solution [1 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactoside in 0.1 M potassium phosphate, pH 7.3, 0.4 M $K_4Fe(CN)_6 \cdot 3\ H_2O$ (13)]. After staining, tissue sections were rinsed in 100 mM Na phosphate, pH 7.3, 2 mM $MgCl_2$, dehydrated successively in 95% and 100% (vol/vol) ethanol and 100% isopropanol, cleared in Histo-Clear, and counterstained with eosin. Tissue fragments were stained similarly, except that fix and wash steps before staining were extended to 15 min and wash buffers contained additionally 0.02% (vol/vol) NP-40 and 0.01% (wt/vol) Na deoxycholate. Stained tissue fragments were finally subjected to three 15-min washes in 3% (vol/vol) dimethyl sulfoxide in PBS (150 mM NaCl, 15 mM Na phosphate, pH 7.3) and three rinses in 70% (vol/vol) ethanol, before storage in 70% (vol/vol) ethanol. Tissue fragments and sections were photographed on Kodak Ektachrome 64T or Gold 100 film, through either a Leitz Laborlux S, or an Olympus SZX binocular light microscope.

In Situ Hybridization

Paraffin sections (7 μm) were mounted on TESPA-treated microscope slides and hybridized at 50 C for 18 h to ^{35}S-labeled sense or antisense RNA transcripts, made either from a template comprising the first 1250 bp of the 5′-end of the *E. coli* β-gal coding region, or a 21-OHase cDNA, and reduced to an average size of 100 nucleotides by hydrolysis in $NaHCO_3$ at 60 C. The hybridization solution contained 50% (vol/vol) formamide, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 μg/ml heparin, 10 mM dithiothreitol (DTT), 0.5 mg/ml sheared herring sperm DNA, 0.5 mg/ml tRNA, 10% (wt/vol) polyethylene glycol 7500, and 1 × Denhardt's solution. After hybridization, slides were washed successively in 2 × SSC, 20 μg/ml RNase containing 0.5 M NaCl and 10 mM DTT, 2 × SSC containing 50% (vol/vol) formamide and 10 mM DTT, and 1 × SSC containing 14 mM DTT and 0.066% (wt/vol) sodium pyrophosphate. Dried slides were dipped in Kodak NTB-2 autoradiography emulsion and exposed for 21–28 days. After developing in Kodak D-19 diluted 1:1 with water, sections were counterstained with methyl green, mounted, and photographed under dark field illumination using Kodak Technical Pan F 2415 black and white film.

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