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Expressed by the Urogenital Sinus Epithelium

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The prostate gland develops from the urogenital sinus by a testosterone-dependent process of ductal morphogenesis. *Sonic hedgehog* (*Shh*) is expressed in the urogenital sinus epithelium and the time course of expression coincides with the formation of the main prostatic ducts. Expression is most abundant in the lumen of the urogenital sinus and in the contiguous proximal duct segments. The initial upregulation of *Shh* expression in the male urogenital sinus depends on the presence of testosterone. The function of Shh was examined in the male urogenital sinus which was transplanted under the renal capsule of an adult male host mouse. Blockade of Shh function by a neutralizing antibody interferes with Shh signaling and abrogates growth and ductal morphogenesis in the transplanted tissue. These observations show that testosterone-dependent *Shh* expression in the urogenital sinus is necessary for the initiation of prostate development.

***Key Words: Shh;* morphogenesis; prostate; testosterone.**

INTRODUCTION

Conserved mechanisms of mesenchymal–epithelial signaling and morphogenesis operate at many different sites in the developing vertebrate embryo. The roles of Hox genes and *Sonic hedgehog* (*Shh*) in development of the limb and central nervous system have been well described (Echelard *et al.*, 1993; Duboule, 1994; Ekker *et al.*, 1995; Nelson *et al.*, 1996; Roelink *et al.*, 1995). Evidence for similar mechanisms of development in soft tissues such as the gut, lung, and genitourinary tract is emerging (Izpisúa-Belmonte *et al.*, 1992; Walterhouse *et al.*, 1993; Roberts *et al.*, 1995; Yokouchi *et al.*, 1995; Bitgood and McMahon, 1995; Benson *et al.*, 1996; Cardoso *et al.*, 1996; Yang *et al.*, 1997; Bellusci *et al.*, 1997). Genes of the Hox A and Hox D clusters have been shown to be expressed in regionalized domains along the axis of the genitourinary tract and transgenic animals with loss of function of single Hox A and Hox D genes have been shown to exhibit both homeotic transformations and dysmorphogenesis of urogenital structures (Dollé *et al.*, 1991; Hsieh-Li *et al.*, 1995; Benson *et al.*, 1996; Kondo *et al.*, 1996; Warot *et al.*, 1997; Podlasek *et al.*, 1999a, b). We have shown previously that the most 5′ member of the Hox D cluster, *Hoxd-13*, is expressed in the urogenital sinus during the period of prostate development (Oefelein *et al.*, 1996) and that loss of *Hoxd-13* function results in a diminished number of main prostatic ducts and decreased prostate size (Podlasek *et al.*, 1997). Recently, Warot *et al.* (1997) observed that mice with compound mutations of the paralogous genes *Hoxd-13* and *Hoxa-13* exhibited severe genitourinary phenotypes, including agenesis of some of the male accessory sex organs.

The *Shh* gene encodes a secreted glycoprotein which is expressed at many sites in the early embryo, including the notochord, floor plate, and limb (Martí *et al.*, 1995; Roberts *et al.*, 1995; Roelink *et al.*, 1995). *Shh* is also expressed at sites of mesenchymal–epithelial interaction in the embryonic lung, hindgut, and genitourinary tract (Bitgood *et al.*, 1995). Genetically engineered loss of *Shh* function produces severe developmental defects in neural and skeletal development, growth inhibition, and embryonic lethality (Chiang *et al.*, 1996). Insight into the function of *Shh* in development of soft tissue structures is limited by prenatal mortality, but several investigators have provided evidence that *Shh* participates in the cascade of molecular signaling between tissue layers during soft tissue morphogenesis. Roberts *et al.* (1995) showed that ectopic expression of *Shh* in the developing hindgut induced expression of downstream targets such as *Bmp4* and *Hoxd-13*. Bellusci *et al.*

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(1996) found that transgenic overexpression of *Shh* in the epithelium of the developing lung increased both epithelial and mesenchymal proliferation. Recently, loss of *Shh* function was shown to produce severe defects in foregut development (Litingtung *et al.*, 1998). We report here that *Shh* is expressed during development of the prostate gland from the male urogenital sinus and provide direct evidence that *Shh* is required for initiation of prostate development.

EXPERIMENTAL

RNA isolation and RT-PCR. Total RNA was isolated from pooled tissue specimens using the TRIzol (Life Technologies) method, and the RNA was DNase treated and resuspended in water as described previously (Podlasek *et al.*, 1997). Nonquantitative RT-PCR was performed with 35 cycles of amplification (Perkin–Elmer RT-PCR Core Kit). Reactions were routinely performed without reverse transcriptase to demonstrate the RNA dependence of the reaction products. Quantitative RT-PCR was performed by determining the ratio between the products for *Shh* and those for the ribosomal subunit *RPL-19*, which was used as an endogenous internal standard. RT-PCR was performed on 50 ng of total RNA using the following program: 42°C for 30 min, 99°C for 5 min, 4°C for 5 min. Equivalent amounts (2.0 μg) of primers for both genes were added to a master mix of PCR reagents (2.5 units AmpliTaq, a final concentration of 2 mM $MgCl_2$, and water). Forty-five-microliter aliquots were placed in five tubes and amplification was performed as follows: 95°C for 2 min and cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min. Tubes were removed at 20, 23, 26, 29, and 32 cycles and samples were electrophoresed on a 1.2% agarose gel (Mallinckrodt; pulsed-field GenAR agarose) containing 50 μg ethidium bromide. Densitometry of the photographed gel was performed using a PDI densitometer. The log of band density vs cycle number was plotted to find the linear range for both products and then the ratio of the products was determined within the linear range. All experiments were performed on pooled tissue specimens. Assays were performed in triplicate and the product ratios reported as the mean plus or minus the standard error of the mean. When determined in this fashion the ratio of the products is independent of the input RNA concentration and is a reliable indicator of relative abundance of expression (Seibert, 1993). The 260-bp *Shh* product was confirmed by restriction digestion with *Msp*I and *Hae*III. Separated mesenchyme and epithelium of day 16 of gestation (E16; plug = day 0) male and female urogenital sinus was kindly supplied by Dr. Gerald Cunha.

RT-PCR primer sequences 5′–3′ were *Shh* antisense, ACTGCTCGACCCTCATAGTG; *Shh* sense, GGCAGATATGAAGGGAAGAT; *RPL-19* antisense, GACAGAGTCTTGATGATCTC; *RPL-19* sense, TCAGGCTACAGAAGAGGCTT; *Ihh* antisense, TCCTGGCTTTACAGCTGACA; *Ihh* sense, CAGAAATGTGATACTCCCGG; *Dhh* antisense, TTCAGTCACACGTAGGCTGA; *Dhh* sense, CACGGAGACGATATGTTCGT; *Ptc* antisense, TACCTAGGAGGTATGCTGTC; *Ptc* sense, GAAGGCGCTAATGTTCTGAC; *FGF-1* sense, GAGGAATGTCTGTTCCTGGA; *FGF-1* antisense, GATGGCTTTCTGGCCATAGT; *Gli* sense, TGCCAGATATGCTTCAGCCA; and *Gli* antisense, TGTGGCGAATAGACAGAGGT.

Immunohistochemistry. Immunohistochemical staining of the E17 male urogenital sinus was performed on sections of formalin-fixed tissue. The primary antibody (Ab80), an affinity-purified rabbit antiserum to amino acids 44–143 of mouse Sonic hedgehog (kindly supplied by Dr. Andrew McMahon), was applied at a concentration of 0.3 μg/ml. Control sections were incubated with rabbit IgG at the same concentration. Staining was performed with 3,3′-diaminobenzidine (DAB) and hematoxylin.

In order to conserve the neutralizing antibody, Ab80, immunohistochemical staining of E15, E18, E19, P5, and P10 urogenital tissue was performed on formalin-fixed sections using a commercially available goat polyclonal IgG antibody (Santa Cruz, Cat. No. sc-1194; 200 μg/ml) to amino acids 30–48, as the primary antibody. According to the manufacturer, this antibody maps to the amino terminus of the Sonic hedgehog protein and does not cross-react with Desert hedgehog or Indian hedgehog. Control sections were incubated with purified goat IgG at the same concentration. The secondary antibody was a biotinylated rabbit anti-goat IgG. Staining was performed with Sigma Fast Fast Red TR/Naphthol AS-MX substrate (Sigma F 4648) and counterstained with hematoxylin.

Organ culture. Organ culture of E15 male urogenital sinus was performed using previously described conditions (Cooke *et al.*, 1987). Briefly, the urogenital sinuses were isolated from the bladder, distal urethra, and Wolffian or Müllerian ducts and cultured on Millicell CM filters (Millipore Corp.) in serum-free medium containing a 1:1 (vol:vol) mix of DMEM:Ham's F-12, 10 ng/ml EGF, 0.01 μg/ml gentamycin, 10 μg/ml insulin, 0.25 μg/ml amphotericin B, 10 μg/ml transferrin, and 5 mg/ml bovine serum albumin (BSA) with or without 10^{-8} M dihydrotestosterone (DHT) in a humidified, 5% CO_2 incubator at 37°C for 72 h. Longer periods of growth under the hormone-supplemented conditions are adequate to support prostatic branching morphogenesis (Podlasek *et al.*, manuscript in preparation; Cooke *et al.*, 1987).

Antibody experiments. Anti-Shh antibody blockade of prostate development was performed using an affinity-purified rabbit polyclonal antibody that recognizes the 19-kDa amino-terminal peptide (Ab80, kindly supplied by A. McMahon; Bumcrot *et al.*, 1995) and that has previously been shown to block Shh-mediated motor neuron induction (Martí *et al.*, 1995). The prostatic anlagen of the E15 male urogenital sinus was isolated, divided in the sagittal plane, and then quartered. The tissue of 8–10 male urogenital sinuses was pooled and then divided equally. Approximately 10–12 Affi-Gel (Bio-Rad) beads equilibrated with PBS were incubated with anti-Shh antibody or rabbit IgG at 3 μg/ml in PBS in a total volume of 5 μl for 1 h. The beads were then combined with the tissue and the tissue–bead mixtures grafted under the renal capsule of the left and right kidneys of an adult male host BALB/c mouse. After growth for 7–10 days the grafts were harvested, photographed, fixed, and sectioned for histology.

Exogenous Shh. Administration of exogenous Shh to the urogenital sinus was accomplished by using CM Affi-Gel Blue beads (200–300 μm in diameter) soaked in purified N-Shh peptide. Affi-Gel beads were equilibrated with PBS and then approximately 60 beads were incubated for 1 h at room temperature in 100 μl of 1.25 mg/ml rat N-Shh 19.6-kDa amino-terminal peptide fragment (kindly provided by Ontogeny, Inc., Cambridge, MA). Control beads were incubated with an equal concentration of BSA. Approximately 20 E15 male urogenital sinuses were isolated and sectioned as described above. The total tissue was combined and then divided into 10 equal parts. The tissue parts were then combined with 6–8 N-Shh-soaked beads (n = 5 samples) or control beads (n = 5). Paired N-Shh/control specimens were then grafted under the capsules of the right and left kidneys, respectively, of a previously castrated adult host (n = 4) or an intact adult male (n = 1). One pair of specimens grafted into a castrated host was retrieved after 3 days

growth. These tissues were used to prepare RNA to determine whether the exogenously administered N-Shh induced *Ptc* expression. The remaining tissue grafts were grown for 10 days before being retrieved, fixed, and sectioned for histologic examination.

RESULTS

Time course of Shh expression in the developing prostate. At E15 the midportion of the male urogenital sinus is a simple tubular structure composed of a multilayered epithelium and a surrounding undifferentiated mesenchyme. Budding of the urogenital sinus epithelium into the surrounding mesenchyme to form the main prostatic ducts begins at E17 in response to testosterone stimulation. Duct formation occurs most intensely in the perinatal period and ceases approximately 10 days after birth. Elongation and branching of the main ducts begins prior to birth, proceeds with maximal intensity through the first 15 days of life, and tapers with approach to maturity (Sugimura *et al.*, 1986). The product of this morphogenetic sequence is an extensively branched ductal system enmeshed in a supporting stroma and connected to the urethra by approximately 25 main ducts. To correlate expression of *Shh* with these morphogenetic events, the time course of expression in the urogenital sinus/prostate was determined by quantitative RT-PCR (Fig. 1, top). A fivefold increase in *Shh* expression between E15 and E18 coincides with the onset of prostate ductal budding. Expression is maintained at this level during the period at which most of the main prostatic ducts are formed (E18 to 5 days postnatal, P5), begins to diminish even while ductal branching is at its zenith (P10–P15), and decreases to a low but detectable level in the adult. The time course of expression shows that an increase in *Shh* expression is temporally associated with the early events of prostate development including formation of the main prostatic ducts and/or branching morphogenesis of the nascent ductal elements.

A previous survey of *Hedgehog* gene expression in the mouse embryo (Bitgood *et al.*, 1995) demonstrated *Shh* expression in the embryonic urethra but did not detect expression of either *Desert hedgehog* (*Dhh*) or *Indian hedgehog* (*Ihh*). When RNA from the E18 urogenital sinus was assayed by RT-PCR using primers specific for these genes, a product signal was not observed for *Dhh* and only a faint product band was observed for *Ihh* at 35 cycles. Examination of *Ihh* expression at several other time points (E15, P5, and P10) revealed expression to be low throughout (data not shown).

Testosterone dependence of Shh expression. The prostate is a unique feature of male sexual development and its morphogenesis from the prostatic anlagen of the urogenital sinus requires the presence of testosterone. The homologous region of the female urogenital sinus, which normally forms the vagina, will undergo prostatic differentiation if exposed to testosterone stimulation (Cunha *et al.*, 1980). To determine whether an upregulation of *Shh* expression is specific to the initiation of prostate development, expression in the female urogenital sinus was examined. A quantitative time course of *Shh* expression in the female shows expression to be low at E15 without significant upregulation of expression at later embryonic time points (Fig. 1, bottom). Comparison of expression in the female and the male at E19 shows significantly greater expression in the male. An obvious explanation for the more abundant *Shh* expression in the male is that *Shh* expression is induced by testosterone. To test whether testosterone is necessary for induction of *Shh* expression, we examined the androgen

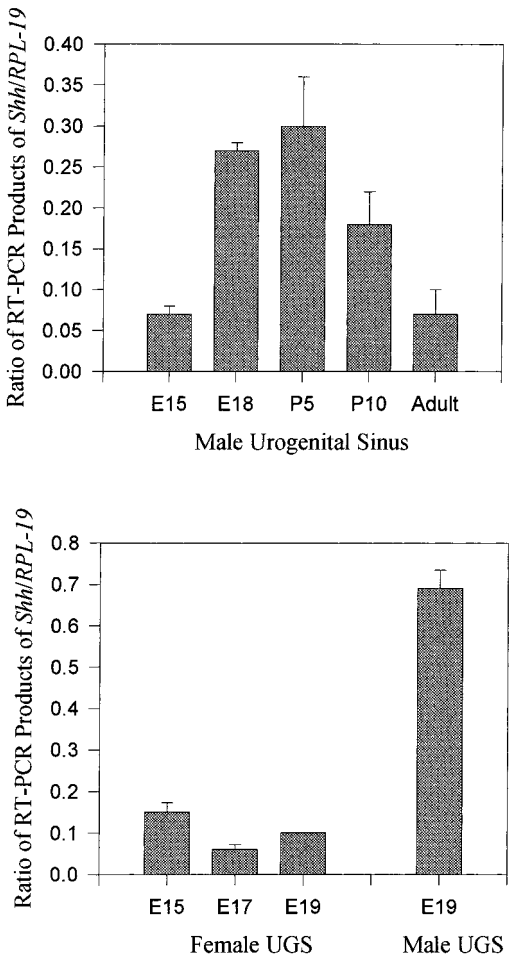


FIG. 1. (Top) Time course of expression of *Shh* in the urogenital sinus and developing prostate as determined by quantitative RT-PCR utilizing message for the ribosomal subunit *RPL-19* as an internal standard. Each time point represents pooled tissue specimens assayed in triplicate; *Shh*/*RPL-19* product ratios are reported as the means plus or minus the standard error of the mean. (Bottom) *Shh* expression in the female urogenital sinus does not increase in late gestation. Comparison of expression in the male and female at E19 shows *Shh* expression to be significantly lower in the female ($P = 0.003$). Each time point represents pooled tissue specimens assayed in triplicate; *Shh*/*RPL-19* product ratios are reported as the means plus or minus the standard error of the mean.

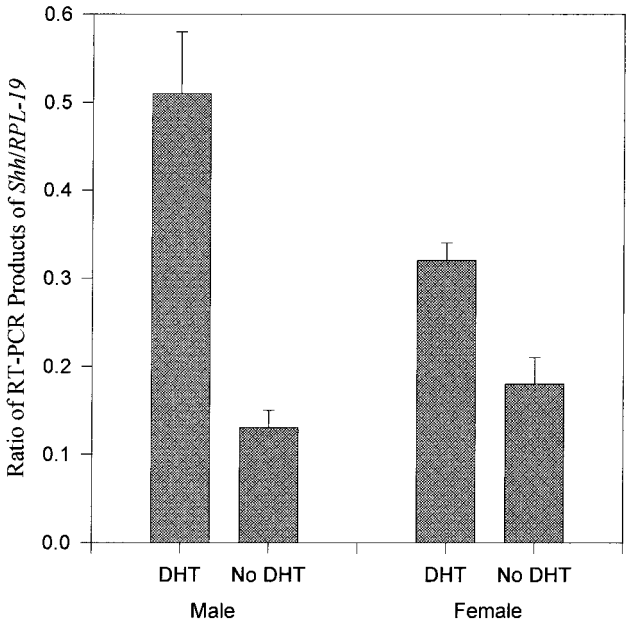


FIG. 2. (Left) Testosterone dependence of *Shh* expression in the male urogenital sinus examined by quantitative RT-PCR of RNA isolated from E15 male urogenital sinus cultured for 3 days in the presence or absence of DHT (10^{-8} M). *Shh* expression is significantly higher in the presence of DHT (unpaired *t* test, *P* value 0.003). Reported values are the results of pooled specimens assayed in triplicate and reported as the means plus or minus the standard error of the mean. (Right) Influence of testosterone on *Shh* expression in the female urogenital sinus examined by quantitative RT-PCR of RNA isolated from E15 female urogenital sinus cultured for 3 days in the presence or absence of DHT (10^{-8} M). *Shh* expression is significantly higher in the presence of DHT (unpaired *t* test, *P* value 0.01). Reported values are the results of pooled specimens assayed in triplicate and reported as the means plus or minus the standard error of the mean.

dependence of *Shh* expression *in vitro*. The E15 male urogenital sinus tissue was cultured *in vitro* for 3 days with and without DHT (10^{-8} M) and quantitative RT-PCR for *Shh* was performed (Fig. 2, left). *Shh* expression was significantly lower in the rudiment cultured in the absence of DHT (unpaired *t* test; $P = 0.003$). When E15 female urogenital sinus was cultured for 3 days in the presence or absence of DHT, *Shh* expression was modestly, but significantly (*P* value 0.01) higher in the presence of DHT (Fig. 2, right). These data suggest that upregulation of *Shh* expression in the male urogenital sinus is induced by testosterone.

Epithelial-specific expression of Shh in the urogenital sinus. The epithelial and mesenchymal tissue layers of the urogenital sinus can be cleanly separated by trypsinization and microdissection (Donjacour *et al.*, 1993). RNA was prepared from separated epithelium and mesenchyme of the E16 male urogenital sinus (kindly provided by Dr. Gerald Cunha) and used for RT-PCR to localize *Shh* expression. A signal attributed to *Shh* expression was detectable in the epithelium but not in the mesenchyme (Fig. 3, top). RT-PCR of RNA prepared from separated epithelium and mesenchyme of the E16 female urogenital sinus also showed localization of *Shh* expression in the epithelium (Fig. 3, bottom).

Immunohistochemical localization of the Shh peptide. The *Shh* gene product is proteolytically cleaved into a 19-kDa amino peptide and a 27-kDa carboxyl peptide, both of which are secreted. The 27-kDa product diffuses freely but the 19-kDa fragment, which appears to be the biologically active moiety, is retained at or near the surface of the secreting cell (Bumcrot *et al.*, 1995; Martí *et al.*, 1995; Porter *et al.*, 1996). Immunohistochemical staining was performed to localize the Shh peptide in the urogenital sinus at the time at which prostate ductal morphogenesis is

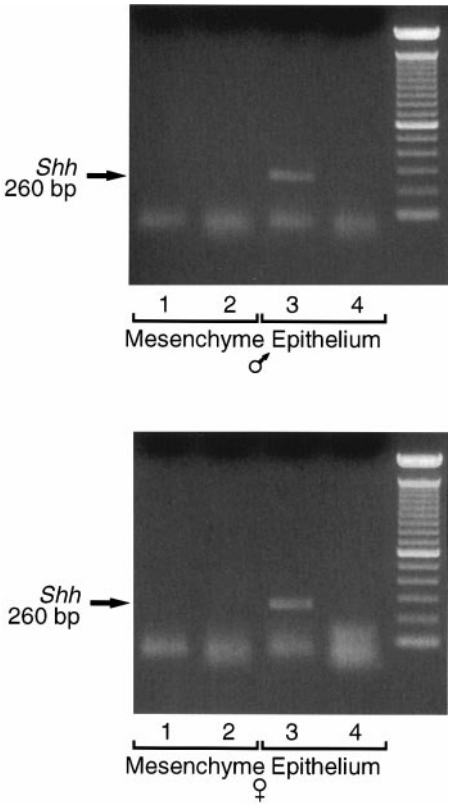
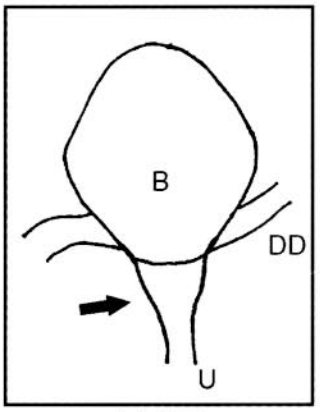
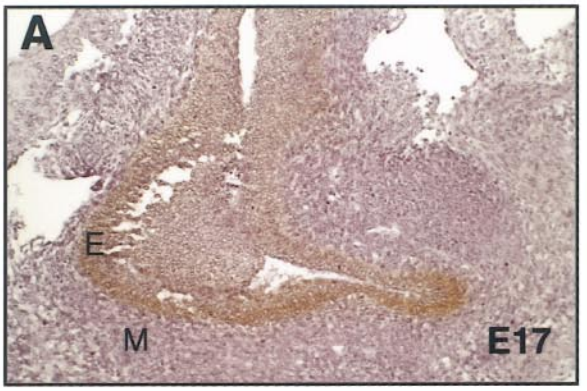
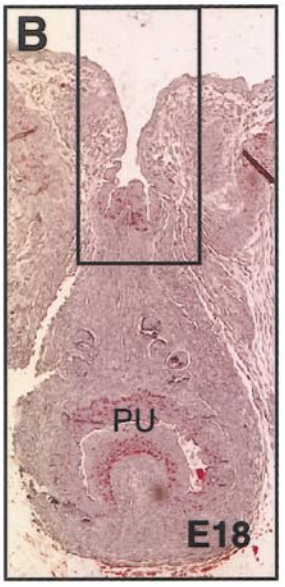


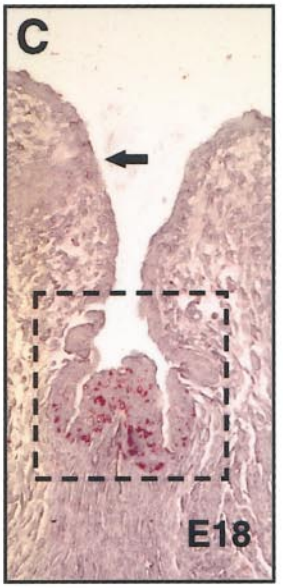
FIG. 3. (Top) Localization of *Shh* expression to the epithelium by RT-PCR prepared from separated mesenchyme (1,2) and epithelium (3,4) of the E16 male urogenital sinus with (1,3) or without (2,4) reverse transcriptase (RT). A hundred-base-pair ladder is shown adjacent. The 260-bp *Shh* PCR product appears uniquely in the reactions containing epithelial RNA and RT. (Bottom) Localization of *Shh* expression in the female urogenital sinus epithelium by RT-PCR of RNA prepared from separated mesenchyme (1,2) and epithelium (3,4) of the E16 urogenital sinus with (1,3) or without (2,4) reverse transcriptase. The 260-bp *Shh* PCR product appears uniquely in the reactions containing epithelial RNA and RT.

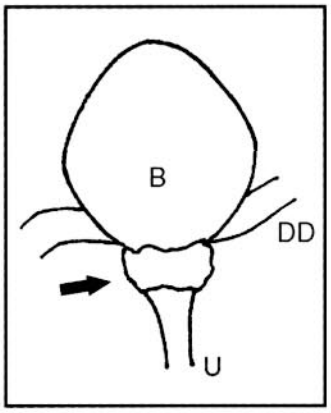


E17

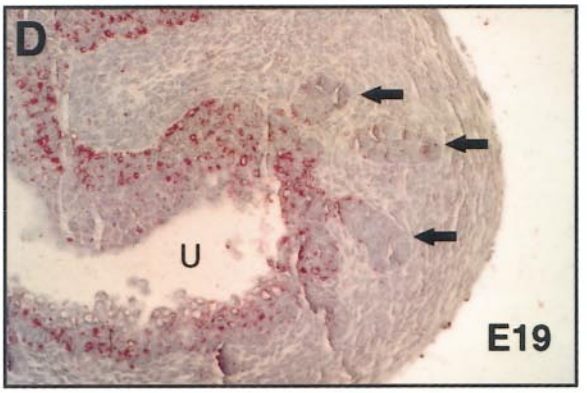


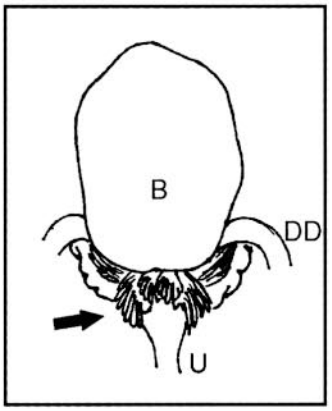




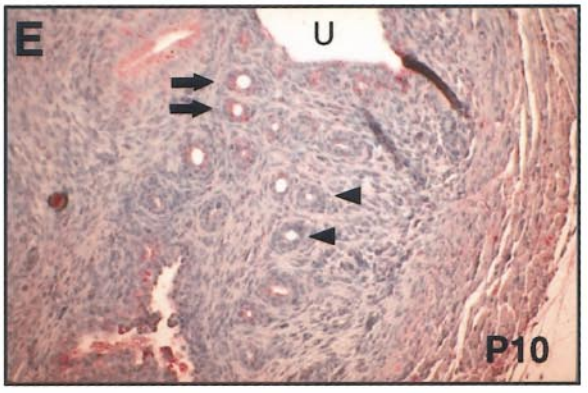


E18-E19





P10



initiated (E17). Immunostaining was performed using an antibody (Ab80) that recognizes the amino terminus of the full-length Shh protein and the biologically active 19-kDa peptide (Bumcrot *et al.*, 1995). Staining in the E17 urogenital sinus was localized to the epithelium (Fig. 4A). No staining was observed in the mesenchyme. Immunostaining with a different anti-Shh antibody (Santa Cruz) that also recognizes the amino terminus was performed at E15, E18, E19, P5, and P10. Staining at E18 was observed in the prostatic urethra but not in the bladder urothelium (Fig. 4B). The transition point is the proximal urethra, just inferior to the junction with the bladder neck (Fig. 4C). Staining at E19 showed a nonuniform pattern of expression within the developing prostate. There was strong staining of the epithelium of the urethral lumen but reduced staining of the epithelium in the nascent prostate ductal buds (Fig. 4D). Regional heterogeneity was also apparent at later time points. Staining at P5 and at P10 (Fig. 4E) revealed Shh to be more abundant in the duct segments closest to the urethra.

Inhibition of Shh function by antibody blockade. The prostatic anlagen of the E15 urogenital sinus can be grafted and grown under the renal capsule of a syngeneic adult male host mouse. This model system has been used extensively to examine the morphogenetic interactions during prostate development since the transplanted tissue undergoes prostatic ductal morphogenesis and terminal prostatic differentiation (Cunha *et al.*, 1987; Donjacour *et al.*, 1993). The role of Shh in prostate development was examined by blockade of Shh function using an affinity-purified antiserum (Ab80) to the N-terminal Shh peptide that is capable of blocking Shh-mediated motor neuron induction in the central nervous system (Martí *et al.*, 1995). Affi-Gel beads were used to absorb the anti-Shh antibody or control antibody and were then implanted along with grafted tissue to serve as a slow release antibody reservoir. To test whether the antibody, delivered in this fashion, effectively interferes with Shh signaling, we examined *Ptc* expression in implanted E15 male urogenital sinus tissue grown for 4 days in the presence of anti-Shh antibody (Ab80) or control antibody beads. *Patched* (*Ptc*) is a gene which encodes a receptor for

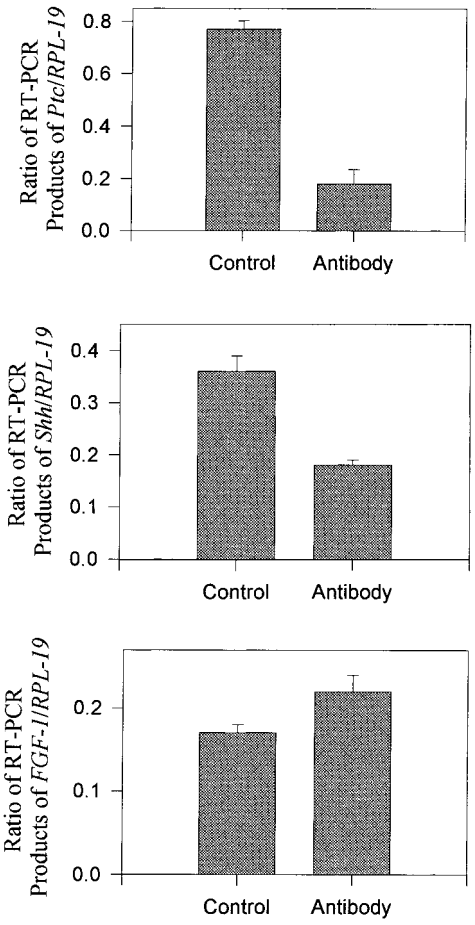


FIG. 5. (Top) Quantitative RT-PCR for *Ptc* was performed on RNA isolated from E15 male urogenital sinus that had been grown under the kidney capsule of an adult male host for 4 days, in the presence of anti-Shh antibody (Ab80) or control antibody. *Ptc* expression is significantly downregulated in the presence of Ab80 (unpaired *t* test, *P* value 0.015). (Middle) Assay of *Shh* expression revealed a decrease in *Shh* expression also ($P = 0.013$). (Bottom) *FGF-1* expression was not significantly altered ($P = 0.65$). Reported values are the results of pooled specimens assayed in triplicate and reported as the means plus or minus the standard error of the mean.

FIG. 4. Immunostaining of formalin-fixed sections of the developing prostate. Schematic diagrams at left show the appearance of the prostate (arrow) prior to the onset of ductal budding (E17), early in the formation of the prostate ductal buds (E18–19), and when significant ductal morphogenesis has occurred (P10). Abbreviations: B, bladder; U, urethra; and DD, ductus deferens. (A) Section of the E17 male urogenital sinus immunostained with an anti-Shh antibody which recognizes both the full-length peptide and the 19-kDa amino-terminal peptide (Ab80, kindly supplied by A. McMahon) stained with DAB and counterstained with Gill's hematoxylin. The anti-Shh antibody specifically stains the urogenital sinus epithelium (E, urogenital sinus epithelium; M, urogenital sinus mesenchyme). An adjacent section stained with control rabbit IgG showed no staining (not shown). (B and C) E18 male urogenital sinus immunostained with a goat polyclonal IgG anti-Shh antibody (Santa Cruz Biotechnology, Inc., Cat. No. sc-1194) whose epitope corresponds to amino acids 30–48 and is also specific for the amino terminus of mouse Shh. (B) Section of the bladder neck (boxed region) and prostatic urethra (PU) of the E18 male. Shh staining is localized to the region of the urogenital sinus which later develops into the prostate. (C) Magnified bladder neck region showing staining is present in the epithelium of the proximal prostatic urethra but absent in the bladder urothelium (arrow). (D) Immunostained section from an E19 male shows stronger staining of the epithelium of the urethral lumen (U) than the nascent prostatic ducts (arrows). (E) Postnatally, proximal duct segments (closest to the urethra) are preferentially stained. In this section of the P10 prostate, arrows highlight stained proximal ducts and arrowheads indicate unstained distal ducts for comparison. The lumen of the urethra is indicated (U).

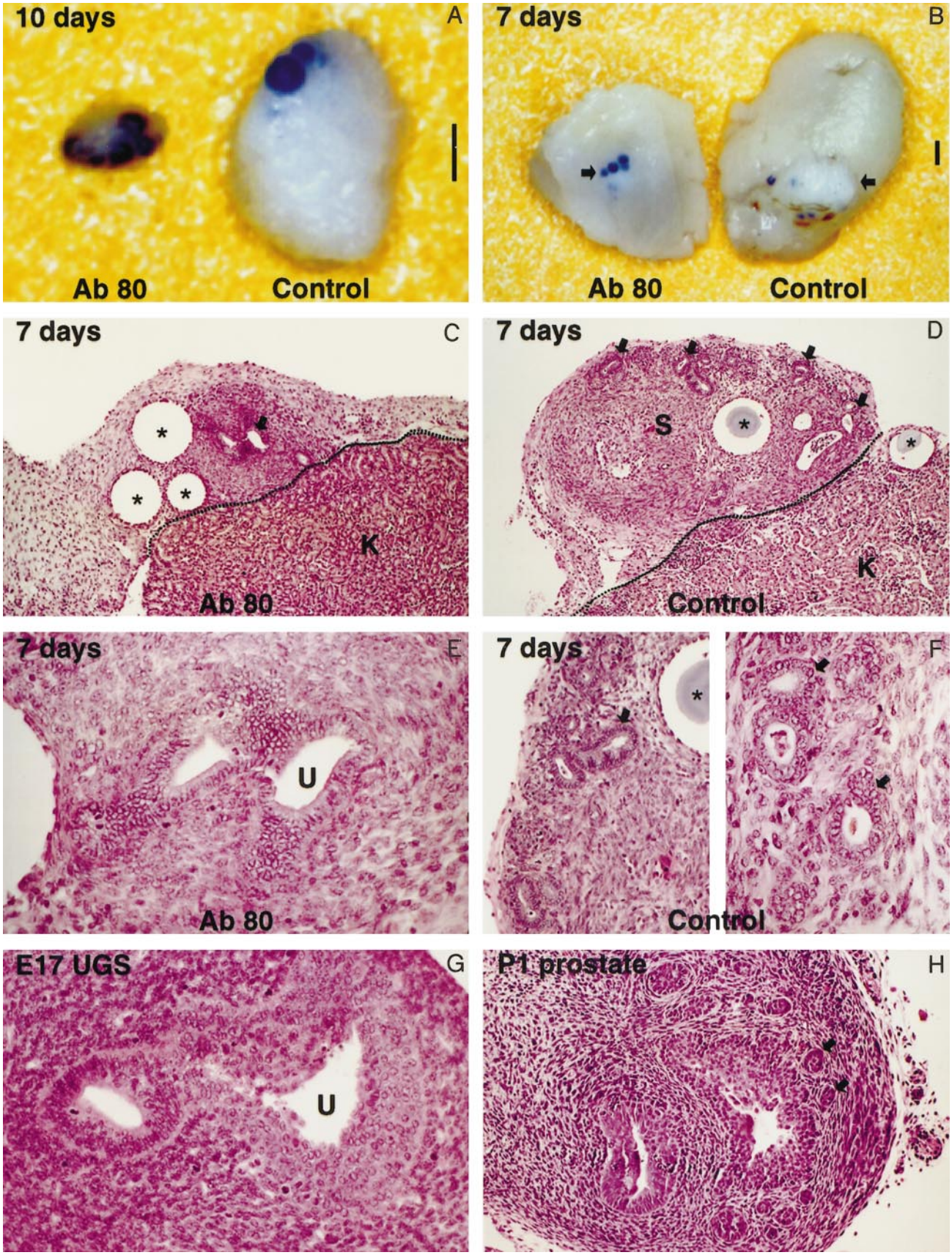


TABLE 1
Inhibition of Ductal Morphogenesis by Anti-Shh Antibody

	Number of ductal lumina	
Days growth	Control	Anti-Shh
7 days growth	11	0
	10	0
	3	0
	1	2
	8	0
	5	0
	7	0
10 days growth	16	3
	16	2
	8	2
	16	1
	38	1

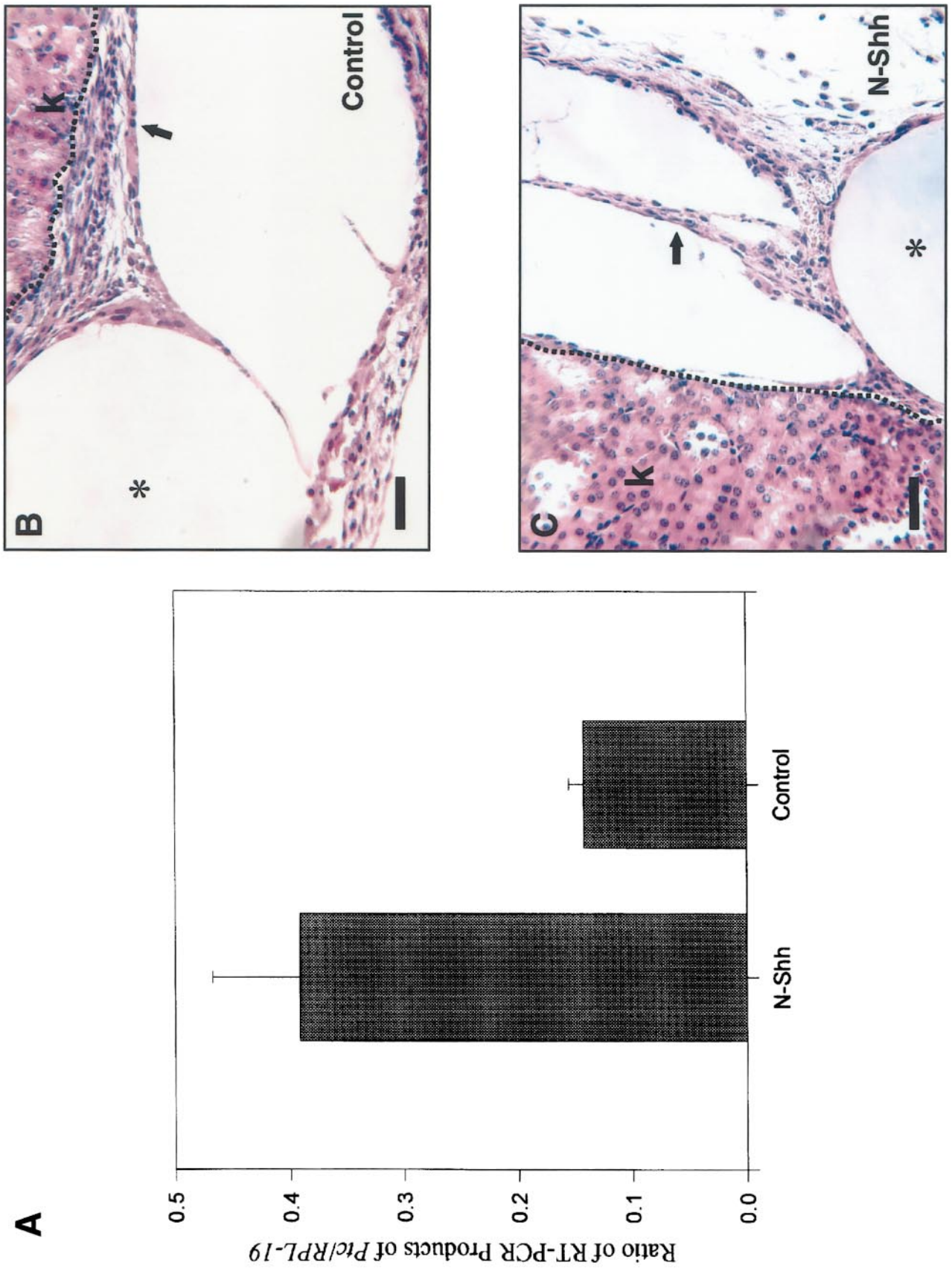
Note. Pooled urogenital sinus tissue from 8 to 10 E15 males were divided, combined with Affi-Gel beads presoaked with the neutralizing anti-Shh antibody Ab80 or control antibody, and grafted under the capsule of the right and left kidneys, respectively, of an adult syngeneic male. Tissues were serially sectioned after growth for either 7 or 10 days and every 10th section was scored for ductal lumina by a blinded observer. Using the number of ductal lumina as an index of ductal morphogenesis, this comparison reveals a significant inhibition of ductal morphogenesis by the anti-Shh antibody ($P = 0.004$; two-way ANOVA).

Shh (Stone *et al.*, 1996). *Ptc* is a target of Shh signaling and an increase in *Ptc* expression is considered a reliable indicator of Shh function (Marigo *et al.*, 1996; Marigo and Tabin, 1996). PCR analysis of expression in the separated mesenchyme and epithelium of the E16 urogenital sinus showed *Ptc* expression primarily localized to the mesenchyme (unpublished observations). When *Ptc* expression was examined in the antibody-treated and control tissues, we found *Ptc* expression was significantly decreased in the presence of Ab80 (Fig. 5, top; $P = 0.015$ unpaired *t* test). This shows that the antibody effectively interferes with Shh signaling. The expression of *Shh* was also slightly decreased by anti-Shh antibody blockade but expression of *FGF-1*, examined to ensure that antibody blockade was not causing a generalized decrease in the expression of non-housekeeping genes, was equivalent in the antibody-treated and control tissues (Fig. 5, middle and bottom, respectively).

Grafted tissues were grown for 7–10 days to examine the effect of Shh blockade on prostate development. The E15 urogenital sinus tissue grafted in the presence of control antibody showed an approximate 10-fold increase in size after 7–10 days. In comparison, the urogenital sinus tissue grafted in the presence of anti-Shh antibody exhibited little or no increase in size (Figs. 6A and 6B). The gross difference in size was attributable to an inhibition of stromal and epithelial proliferation by the blocking antibody. Histologic sectioning of the tissue grafted in the presence of the blocking antibody revealed a small tissue mass with scant stroma and an absence of ductal morphogenesis. Typically, only a simple tubular lumen lined by a multilayered epithelium and surrounded by a thin stromal sheath was observed (Figs. 6C and 6E). This appearance is essentially unchanged from the appearance of the male urogenital sinus prior to the onset of ductal morphogenesis (E17 urogenital sinus shown for comparison, Fig. 6G). In contrast, histologic sectioning of tissue grafted in the presence of the control antibody revealed multiple ductal elements enmeshed in an abundant stroma (Figs. 6D and 6F). This appearance is similar to the normal newborn prostate shown for comparison (Fig. 6H). Quantitative analysis of ductal morphogenesis in grafted tissues confirmed a nearly complete inhibition of growth and ductal morphogenesis by the anti-Shh antibody (Table 1).

In order to determine whether Shh is sufficient to initiate prostate development in the absence of testosterone, we examined the ability of exogenously applied Shh to induce glandular morphogenesis in the urogenital sinus in an

FIG. 6. (A and B) Inhibition of growth in the urogenital sinus demonstrated by antibody blockade of Shh function. Pooled tissue from E15 male urogenital sinuses ($n = 8$) were sharply divided and each half was combined with 10–12 Affi-Gel Blue beads preincubated with either anti-Shh antibody (Ab80) or control rabbit IgG at 3 μg/ml in PBS for 1 h. (A) Specimens were harvested from under the renal capsule after 10 days of growth. Blue beads are Affi-Gel. Comparison of tissue grafted with the Shh antibody (left) and with the control antibody (right) show a marked difference in size. Growth appears to be inhibited by the Shh antibody. Size marker, 1 mm. (B) Tissues plus beads were grown for 7 days. Segments of kidney are shown with the tissue grafts (arrows) in place beneath the renal capsule. Size marker, 1 mm. Apparent inhibition of growth by the anti-Shh antibody (left) is clearly seen. (C–H) Histologic section of tissue grown for 7 days in the presence of Shh antibody reveals a simple tubular lumen (C, 100× and E, 200×). Note the absence of any small ductal structures or other evidence of ductal morphogenesis. The lumen is indicated (U). Bordering kidney tissue (K) is demarcated by a dashed line; cystic spaces created by the Affi-Gel beads are marked with an asterisk. This appearance is indistinguishable from the normal E17 urogenital sinus shown for comparison (G, 200×). Histologic sectioning of tissue grown for 7 days in the presence of control antibody shows an overall greater tissue mass, composed of both stroma (S) and multiple ductal elements (arrowheads; D, 100× and F, left, 200× and right, 400×). Note the single layer epithelium of the nascent prostatic ducts. The newborn mouse prostate is shown for comparison (H, 200×). Histologic sectioning of control tissues grown *in situ* for 24 days after grafting revealed characteristic prostate ductal morphology; prostatic differentiation was confirmed by Alcian blue staining (not shown).



androgen-deprived environment. CM Affi-Gel beads soaked in 1.25 mg/ml purified N-Shh peptide or control beads were combined with urogenital sinus tissues and grafted under the capsule of the right and left kidneys, respectively, of previously castrated adult male mice ($n = 4$) or an intact adult male ($n = 1$). The concentration of N-Shh used has been shown to be sufficient to induce *Shh* target gene expression and produce morphologic changes in both the developing tooth and the limb bud (Yang *et al.,* 1997; Hardcastle *et al.,* 1998). One set of tissues grafted in a castrated host was retrieved after 3 days growth and used to prepare RNA. Quantitative RT-PCR confirmed an increase in *Ptc* expression in the tissue grown with Shh-soaked beads compared to the tissue grown with control beads (Fig. 7A; $P = 0.03$). Expression of another conserved target of Shh signaling, *Gli,* was also significantly increased (data not shown). These data confirm that the beads delivered physiologically active concentrations of N-Shh. The remaining tissues were examined histologically after 10 days growth. The urogenital sinus tissues grafted in the intact male displayed glandular morphogenesis characteristic of the developing prostate (not shown). Tissues grafted with control beads in the castrated animals showed a complete absence of glandular morphogenesis and a flattened epithelium (Fig. 7B), consistent with vaginal differentiation (Cunha *et al.,* 1977). The tissues grafted with Shh-soaked beads exhibited that same appearance (Fig. 7C). Both control and Shh-treated tissues showed complete absence of glandular morphogenesis. These observations suggest that Shh is not sufficient to induce prostate development in the absence of testosterone.

DISCUSSION

The signaling cascade which mediates interaction between the epithelium and the mesenchyme in prostate morphogenesis is as yet largely unknown, but it is the focus of intense interest because of its potential relevance to human prostatic disease. Studies of embryonic development in several organ systems have revealed an apparently conserved mechanism of mesenchymal–epithelial signaling related to the *hedgehog–decapentaplegic* signaling pathway of *Drosophila.* A survey of *Shh* expression in the developing embryo showed *Shh* to be expressed at numerous sites of mesenchymal–epithelial interaction. Expression is seen predominantly in the epithelium of the tooth, hair, whisker, rugae, bladder, urethra, vas deferens, gut, and lung (Bitgood and McMahon, 1995). An influence of *Shh* on proliferation and growth has been shown. Overexpression of *Shh* in the developing limb increased cell proliferation (Scott *et al.,* 1997). Similarly, overexpression of *Shh* in the developing lung resulted in increased mesenchymal and epithelial cell proliferation at E16.5 and E17.5 (Bellusci *et al.,* 1997). A survey of expression of the *decapentaplegic* homologs *Bmp2* and *Bmp4* in the developing embryo showed expression present in tissues adjacent to areas of *Shh* expression (Bitgood and McMahon, 1995). This was interpreted as suggesting a conserved mechanism of mesenchymal–epithelial signaling in soft tissue morphogenesis. Consistent with this hypothesis, ectopic expression of *Shh* in the hindgut was shown to induce mesodermal *Bmp4* expression (Roberts *et al.,* 1995). *Hoxd-13* appears to be another target of *Shh* signaling in some regions. In the limb bud and hindgut, *Hoxd-13* expression is associated with *Shh* during development and can be induced by ectopic *Shh* expression (Riddle *et al.,* 1993; Roberts *et al.,* 1995). Both *Bmp4* and *Hoxd-13* have been shown to influence growth during morphogenesis (Dollé *et al.,* 1991; Francis *et al.,* 1994; Kondo *et al.,* 1996). Taken together, these observations suggest a pivotal role for *Shh* in a signaling pathway that involves both mesenchyme and epithelium and results in expression of genes with demonstrated effects on growth (Sordino *et al.,* 1995; Blessing *et al.,* 1993; Bellusci *et al.,* 1996; Duprez *et al.,* 1996).

We have shown that the *Shh* signaling pathway is operative and necessary in the developing prostate. It is therefore relevant that two targets of *Shh* signaling, *Hoxd-13* and *Bmp4,* are expressed in the developing prostate. *Hoxd-13* has been shown to positively regulate prostate growth. *Hoxd-13* is expressed in the mesenchyme and epithelium of the urogenital sinus and in the epithelium of the nascent prostatic ducts (Oefelein *et al.,* 1996). Expression parallels morphogenesis of the prostate ductal architecture and is downregulated as ductal morphogenesis is completed. The *Hoxd-13* loss-of-function mutant has dorsal and ventral prostate lobes which are decreased in size, have a reduced number of main prostatic ducts, and show diminished

FIG. 7. Effect of exogenous N-Shh peptide on the urogenital sinus tissue grown as a transplant under the renal capsule of a castrated adult male mouse. Pooled tissue from E15 male urogenital sinuses ($n = 20$) was sharply divided and then separated into 10 equal parts. Each part was combined with 6–8 CM Affi-Gel Blue beads preincubated with 1.25 mg/ml N-Shh or control beads and then grafted under the capsule of the right and left kidneys, respectively, of a previously castrated adult male mouse. One pair of specimens was retrieved after 3 days growth for gene expression analysis. The remainder were grown for 10 days before being retrieved, fixed, and sectioned. (A) Quantitative RT-PCR analysis of *Ptc* expression in tissues grown for 3 days in the presence of N-Shh-soaked beads or control beads shows induction of *Ptc* expression by N-Shh. (B and C) Representative histologic sections of tissues grown with either control beads (B) or N-Shh-soaked beads (C) showing absence of glandular morphogenesis and a flattened epithelium (arrow) consistent with vaginal differentiation. Bordering kidney tissue (k) is demarcated by a dashed line; cystic spaces created by the CM Affi-Gel beads are marked with an asterisk. Size markers indicate 0.1 mm.

ductal branching (Podlasek *et al.,* 1997). We have recently found that *Bmp4* is also expressed in the developing prostate. We have shown that it is expressed primarily in the mesenchyme and exerts an inhibitory influence on epithelial proliferation (unpublished observations).

A classical understanding of the role testosterone plays in prostate development comes from analysis of tissue recombinants. Briefly summarized, these elegant studies have shown that prostatic morphogenesis and differentiation are products of a localized instructive potential in the mesenchyme and a receptive urogenital sinus epithelium (Cunha *et al.*, 1987). Analysis of growth and differentiation in tissue recombinants composed of mesenchyme and epithelium from wild-type and androgen receptor-deficient *Tfm* mice, demonstrated that prostate development depends upon functional androgen receptors in the mesenchyme only. Epithelial androgen receptors are necessary for full prostatic differentiation and secretory function but not for ductal morphogenesis (Donjacour *et al.,* 1993). This influence of testosterone on morphogenesis has been attributed to androgen-stimulated synthesis of mesenchymal factors which regulate epithelial cell proliferation (Shima *et al.*, 1995). Our studies show that Shh is necessary for the initiation of prostate development. Since *Shh* expression is specific to the epithelium, it is surprising that upregulation of expression depends on the presence of testosterone. This observation suggests the presence of a paracrine mechanism which links testosterone action in the mesenchyme to epithelial expression of *Shh*. Our finding that DHT treatment will induce *Shh* expression in the female urogenital sinus is consistent with the experimental observation that the female urogenital sinus will undergo prostatic development when placed in an androgen environment (Cunha and Lung *et al.*, 1980).

Shh has been found to play an inductive role in development of bony structures of the limb bud and central nervous system. *Shh* may also be pivotal to induction of soft tissue morphogenesis. Indeed, the finding of severe defects of foregut development in the Shh loss-of-function mouse mutant is consistent with such a role (Litingtung *et al.*, 1998). We propose that *Shh* occupies a critical place early in the mesenchymal–epithelial signaling cascade of prostate development and that upregulation of *Shh* expression by testosterone is a critically important event in the initiation of prostate development. The inability of exogenously supplied Shh to induce glandular morphogenesis in the absence of testosterone, despite activation of Shh target genes, suggests that the requirement for testosterone in prostate development extends beyond induction of *Shh* expression.

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