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Expression of murine renin genes in subcutaneous connective tissue

(large tumor antigen/immunohistochemistry/transgenic mice/eviscerated fetus/development)

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ABSTRACT **A renin promoter–large tumor antigen (T antigen) fusion gene was constructed to provide a reporter function for renin expression in transgenic mice. These transgenic mice gave rise to tumors in subcutaneous soft tissue, which was attributed to transgene expression at this site. An immunohistochemical analysis of transgenic fetuses from several independent lines revealed scattered T-antigen-containing mesenchymal cells and fibroblasts in the subcutaneous layer of the skin between the panniculus carnosus muscle of the skin and the skeletal muscle of the body wall. This localization is consistent with the location of overt tumorigenesis in adult mice. This pattern was specific for the renin–T antigen fusion gene as no immunohistochemical staining was observed in transgenic fetuses containing a heterologous promoter–T antigen fusion gene. Northern blot analysis of tumor RNA indicated that most of the tumors expressed both T antigen and the endogenous renin gene *Ren-1*^{c}. In addition, when multiple renin genes were introduced by crossing transgenic mice with nontransgenic DBA/2J mice, which contain another allele of the *Ren-1* locus as well as the duplicated locus *Ren-2*, the resultant tumors expressed the *Ren-2* gene. Northern blots were then used to analyze renin expression in the subcutaneous tissue of normal mice. Fully processed renin mRNA was detected in eviscerated 15.5-day postcoitus fetal and newborn carcasses and in newborn skin. Our data indicate that there is a renin-expressing cell population in fetal and newborn subcutaneous tissue.**

Renin is an aspartyl protease that participates in the regulation of blood pressure and electrolyte balance. It catalyzes the first step in the pathway leading to the production of angiotensin II (A-II), which has vasoconstrictor activity and stimulates the production of aldosterone and prostaglandins (1, 2). Although the classical site of synthesis of circulating renin is the kidney, many extrarenal tissues have been found to contain either renin or renin mRNA (3–8). Renin synthesis at many of these sites is conserved through evolution, yet its function at present remains unclear. In addition to renin, other components of the renin–angiotensin system (RAS)—namely, angiotensinogen mRNA, A-II, and angiotensin-converting enzyme—have been reported to exhibit a wide tissue distribution (9–13). Although the RAS has been classically considered to be an endocrine system, the identification of components of the RAS in a wide spectrum of tissues has led to the proposal that there are local or tissue RAS participating in local autocrine and paracrine functions (14).

We chose to investigate further the tissue-specific expression of renin in the mouse in an effort to identify and define previously unreported sites of renin expression. We used an approach in which expression of an indicator gene, in this case the simian virus 40 large tumor antigen (T antigen), was driven by a renin promoter. The utility of T antigen providing the reporter function is twofold. First, its products accumulate in the nucleus and thus are easily detected by immunohistochemistry. Second, the ability of T antigen to transform cells to a tumorigenic phenotype when its expression is specifically targeted to these cells is well documented (15–17) and may help to identify T-antigen-expressing cells even when they constitute only a small fraction of the cells in a tissue. Therefore, we reasoned that a combination of immunohistochemical analysis and T-antigen-induced neoplasia should facilitate the detection of previously unreported sites of renin expression if T-antigen expression could be specifically directed to renin-expressing cells. Here, we report the identification of renin synthesis in fetal and newborn subcutaneous connective tissue.

MATERIALS AND METHODS

Production of Transgenic Mice. The transgene contains 4.6 kilobases (kb) of *Ren-2* 5′ flanking sequence isolated from a DBA/2J genomic clone. A fusion was made between this sequence at +6 relative to the major transcription start site and the simian virus 40 T-antigen structural gene at −74 with respect to the translation initiation codon. The transgene was constructed by first cloning the most proximal region of the *Ren-2* promoter as a 124-base-pair *Bam*HI–*Alu* I fragment into *Bam*HI–*Xba* I sites of pUC18 after converting the *Alu* I site to an *Xba* I site by the use of a linker. The T-antigen gene was then cloned into this plasmid as an *Xba* I–*Sal* I fragment after removing a *Bam*HI site downstream of the T-antigen structural gene. The final plasmid was constructed by ligating a 4.5-kb *Bam*HI fragment containing the 5′ flanking sequence of *Ren-2* (containing nucleotides −4600 to −119) into the above plasmid.

The production of transgenic mice was performed by using established procedures (18) and as previously described (45). Positive transgenic mice were identified by Southern blot hybridization (19) using flanking renin sequences as a probe. Only the T4 founder and the first three of its offspring were inbred C57BL/6Ros. All subsequent T4 offspring and all other lines were C57BL/10Ros × C3H/HeRos (BCF_1) mice.

Expression Studies. RNA from total tumor or fetal tissue was isolated as described (20). For expression studies, RNA was fractionated on a 1.5% agarose gel after denaturation with glyoxal or formaldehyde as described (20). Northern blots were probed for T antigen by using a [^{32}P]GTP-labeled T3 polymerase antisense T-antigen transcript from the plasmid T3T7-TAG (45) and for renin by using a [^{32}P]GTP-labeled

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Abbreviations: T antigen, large tumor antigen; RAS, renin–angiotensin system; A-II, angiotensin II.

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SP6 polymerase antisense renin transcript from the plasmid pSLM (21). Blots containing fetal tissues were incubated in RNase A at 1 μg/ml for 15 min at room temperature to remove nonspecific hybridizing probe.

Locus-specific primer extension reactions were performed as described (5) on tumor RNA isolated from mice bred to contain each of the three inbred renin genes. These mice were produced by crossing transgenic mice (containing the *Ren-1*^{c} gene) with nontransgenic DBA/2J mice (containing the *Ren-1*^{d} and *Ren-2* genes). Immunoperoxidase staining for T antigen was carried out on 10-μm frozen sections of 15.5-day postcoitus transgenic fetuses essentially as described (22).

Whole embryos were cross-sectioned, and 3-mm segments (Fig. 4, lanes 1 and 6) or 1.5-mm segments (Fig. 4, lanes 2–4) were pooled. Sections were taken every 100 μm for histology. To assay for renin expression in subcutaneous tissue, 15.5-day postcoitus fetuses and newborns were decapitated and eviscerated. The carcasses were thoroughly washed in saline to ensure they were free of contaminating viscera.

RESULTS

Tumor Formation in Renin–T Antigen Transgenic Mice. T-antigen expression was induced in renin-expressing cells by fusing its structural gene to 4.6 kb of *Ren-2* 5′ flanking sequence. Adult transgenic mice from five of the original eight transgenic lines exhibited a significant but variable frequency of neoplasia in the kidney, adrenal gland, testis, and submandibular gland, all sites of known *Ren-2* expression. The neoplasias included mild to severe cellular hyperplasias and tumor formation in several cases. No dysplastic or neoplastic growth occurred in any other parenchymal organs. The three remaining transgenic lines have not demonstrated any evidence of tumorigenesis.

In addition to tumors derived from sites previously shown to express *Ren-2*, mice representative of four transgenic lines exhibited soft tissue tumors. The tumors were distributed over the entire body surface but were more frequent in the subcutaneous tissues along the flank and in the back of the neck and, to a lesser extent, in the intraperitoneal soft tissues. The tumors appeared to originate from fascial connective tissue overlying the skeletal muscle of the body wall and on occasion were seen to be locally invasive. They were histopathologically characterized as mixed sarcomas. None of the negative littermates of any transgenic line developed tumors during the observation period, which lasted beyond the age of their positive siblings. About 10% of the mice from the T7 transgenic line, one of the three mice examined from the T3 transgenic line, and the T8 founder (the only mouse isolated from this line) exhibited subcutaneous tumors. The T4 transgenic line presented the highest incidence of subcutaneous tumors (greater than 50%), afforded the largest number of animals for study, and is described in further detail below.

Subcutaneous T-Antigen Expression Prior to Tumorigenesis. We sought to determine the extent of T-antigen expression in subcutaneous tissue prior to the onset of neoplasia in order to correlate transgene expression with tumorigenesis. Therefore, transgenic fetuses were recovered, sectioned, and analyzed for T-antigen accumulation by immunohistochemistry (Fig. 1). T antigen was detected in the adrenal glands and kidneys, consistent with the pattern of fetal *Ren-2* expression (22). T antigen was also detected in cells scattered throughout the developing subcutaneous tissue in regions around the flank (Fig. 1) as well as behind the neck (data not shown), consistent with the localization of tumors in adult T4 animals. More precisely, the T-antigen-containing cells were limited to a specific population of mesenchymal cells and fibroblasts in a region between the panniculus carnosus muscle within the developing skin and the skeletal muscle of the developing body wall. The close association between the T-antigen-containing cells and the developing muscle is evident in some oblique skin sections containing multiple muscle layers (Fig. 1*C*). The T-antigen-immunoreactive cells appear to be localized to regions of developing fascial connective tissue (Fig. 1*C*).

Importantly, the examination of fetal T-antigen accumulation in offspring from two other transgenic lines (T7 and T3) qualitatively demonstrated the same localization of T-antigen-containing cells (data not shown), suggesting that T-antigen expression was not caused by the site of transgene insertion. An analysis of fetal T-antigen expression in transgenic mice containing a mouse major urinary protein promoter–T antigen fusion gene (23) revealed no evidence of T-antigen immunoreactivity in subcutaneous tissue or in any

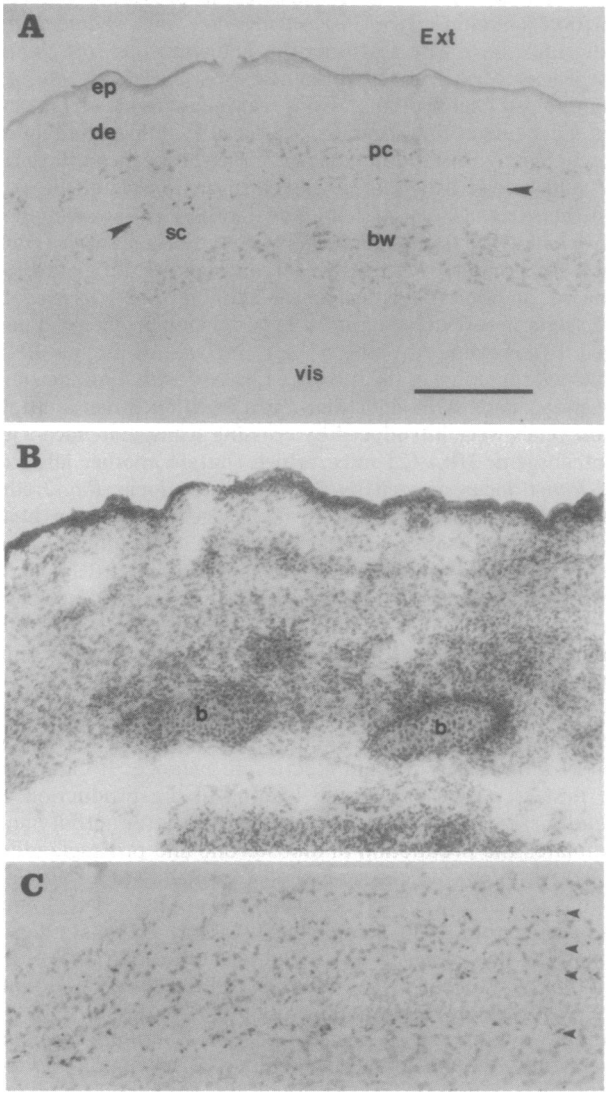

FIG. 1. Fetal T-antigen expression in subcutaneous tissue. A T4 transgenic fetus was frozen in liquid nitrogen and cryosectioned for immunohistochemistry to T antigen. Representative serial coronal sections are shown. (*A*) Layer of T-antigen staining (between arrows) in a specific population of mesenchymal cells in the developing subcutaneous tissue on the flanks. These cells are localized between the panniculus carnosus muscle (pc) and the skeletal muscle of the body wall (bw). The section was lightly counterstained with eosin. (*B*) Serial section stained with hematoxylin/eosin. (*C*) Oblique skin section showing the close association between the T-antigen-containing cells (arrowheads) and the developing muscle. ep, Epidermis; de, dermis; sc, subcutaneous; vis, viscera; Ext, external surface of embryo; b, developing bone. (Bar = 100 μm.) Color photography of this figure is available for inspection upon request.

other site known to express renin at this stage of fetal development, which further demonstrates the specificity of our results (data not shown). The fact that the level of T antigen in the subcutaneous tissue of T4 mice was higher than either T7 or T3 mice may explain the higher incidence of subcutaneous tumors in the T4 line.

Renin Expression in Subcutaneous Tumors. Since renin expression in subcutaneous tissue was not previously identified, it was necessary to distinguish the tumorigenic amplification of bona fide renin-expressing cells from metastasis or ectopic transgene expression. By a careful postmortem examination and histopathological analysis of the transgenic mice, we were able to rule out the possibility of metastasis as a contributing factor to tumorigenesis at this site because (*i*) many mice exhibited only subcutaneous tumors and (*ii*) there was no involvement of regional lymph nodes, the lung, or liver. Therefore, it was concluded that the subcutaneous tumors originated from multicentric primary sites and not secondary metastatic lesions. To address the question of ectopic expression of the transgene, RNA was extracted from subcutaneous tumors, and Northern blots were probed for expression of T antigen and the endogenous renin gene *Ren-1^c*. *Ren-1^c* codes for the classical circulating renin isozyme in strains of mice (BCF) that contain only a single renin gene. Results of this analysis indicated that the subcutaneous tumors from T4 transgenic mice expressed both *Ren-1^c* (Fig. 2*A*) and T antigen (Fig. 2*B*). Several subcutaneous tumors that arose in mice from other transgenic lines were tested and found to express *Ren-1^c* (Fig. 2*C*, lanes 9 and 10). The sizes of the mRNAs for both the transgene and the endogenous renin gene were consistent with proper transcription initiation and RNA processing. By comparison with renin expression at other known sites, we estimated the *Ren-1^c* mRNA content of these cells to be as great as 100 copies per cell (Fig. 2*A*, lane 7). Although T-antigen mRNA was detectable in all tumor RNA samples, the level was highly variable and there was no correlation between the level of T antigen and renin mRNA among individual samples. Renin *in situ* hybridization and T-antigen immunocytochemistry revealed that they were colocalized and restricted to the neoplastic cell population of the tumors (data not shown).

We next introduced another renin gene from an independent locus by crossing T4 transgenics with nontransgenic DBA/2J mice and assayed the offspring for the presence of the transgene by dot blot hybridization. Since DBA/2J mice contain the *Ren-2* gene and a different allele of the *Ren-1* locus, the resultant transgenics were effectively heterozygous for both alleles of the *Ren-1* locus and contained one copy of the *Ren-2* locus. RNAs were purified from the resultant tumors and analyzed by Northern blot hybridization (Fig. 2*C*, lane 11) and by a locus-specific primer extension assay taking advantage of specific nucleotide sequence differences between the highly homologous genes (ref. 5; Fig. 3). These results clearly demonstrated the presence of a *Ren-2*-specific band in addition to the *Ren-1*-specific band in tumor RNA from T4 × DBA mice (Fig. 3, lanes 1 and 2) but not in tumor RNA from the original BCF transgenics (Fig. 3, lane 3), indicating that the tumor cells expressed *Ren-2* and at least one allele of the *Ren-1* locus. Similar results were obtained with other T4 × DBA animals (data not shown). The greater intensity of the *Ren-1*-specific band may be the result of combined expression of both the *Ren-1^c* and *Ren-1^d* alleles, which were not differentiated by this assay, or the result of differential expression of the *Ren-1* and *Ren-2* genes in this tissue. Differential expression of the murine renin genes at several extrarenal tissues has been demonstrated (7, 8). These arguments notwithstanding, the above findings suggest that renin expression in subcutaneous tumors is not caused by isolated cis-acting mutations of the *Ren-1^c* gene.

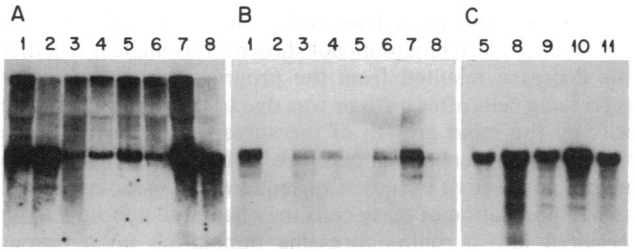

FIG. 2. Northern blot analysis of subcutaneous tumor RNA. (*A*) Twenty micrograms of tumor RNA from the T4 offspring was probed for expression of renin. (*B*) The same samples were probed for expression of T antigen. (*C*) Twenty micrograms of tumor RNA from animals 5 and 8 in *A* and from subcutaneous tumors from a T7 (lane 9), a T3 (lane 10), and a T4 × DBA transgenic mouse (lane 11) was probed for expression of renin.

Renin Expression in Normal (Nontransgenic) Fetal Subcutaneous Tissue and Skin. To determine the extent of renin expression in normal mouse tissues, a 15.5-day postcoitus nontransgenic BCF_2 fetus was collected, and multiple cryosections were pooled into segments for analysis of renin mRNA (Fig. 4). This stage of fetal development was chosen because it is characterized by high levels of renin expression in the kidney and adrenal gland (21, 22). In addition, sections were taken every 100 μm for a histological determination of the tissues contained within each segment. The data demonstrated the presence of renin mRNA in each of the pooled segments. The relative abundance of renin mRNA in segment 1 is due mostly to the presence of the adrenal gland and kidney. Although segments 5 and 6 contained the developing brain, which is thought to be a site of renin expression in adults (24), it is worth noting that they also contained the peripheral tissues that were most susceptible to tumorigenesis in adult transgenics (see above) and had a high concentration of T-antigen-containing cells in 15.5-day postcoitus fetuses.

The above experiments, while showing that a population of renin-expressing cells was present in each segment, could not positively identify which tissues contained within each segment expressed renin. Therefore, we attempted to identify renin mRNA more directly in the subcutaneous tissue of a normal mouse. To accomplish this, RNA was purified from

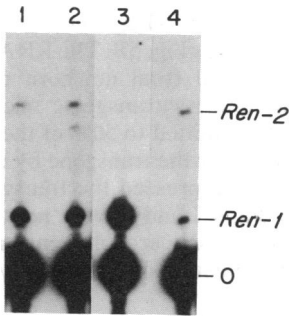

FIG. 3. Locus-specific primer extension of tumor RNA. Eighty micrograms of tumor RNA was hybridized with a 38-base oligonucleotide specific for bases 1031–1068 of the mature *Ren-2* mRNA (5). Primers were extended in the presence of ddCTP and three other NTPs. The unextended oligonucleotide (0), *Ren-1*-, and *Ren-2*-specific bands are shown. Lanes 1 and 2, two independent tumors from T4 × DBA mice. Lane 3, tumor from a transgenic BCF_2 mouse. Lane 4, kidney RNA from a male DBA/2J showing the relative position of the *Ren-1* and *Ren-2* bands. The extra band in lane 1 resulted from premature termination of primer extension, which occurs in some tumor RNA samples. The RNA in lane 1 is from the same sample as in Fig. 2, lane 11. The RNA in lane 3 is from the same sample as in Fig. 2, lane 7.

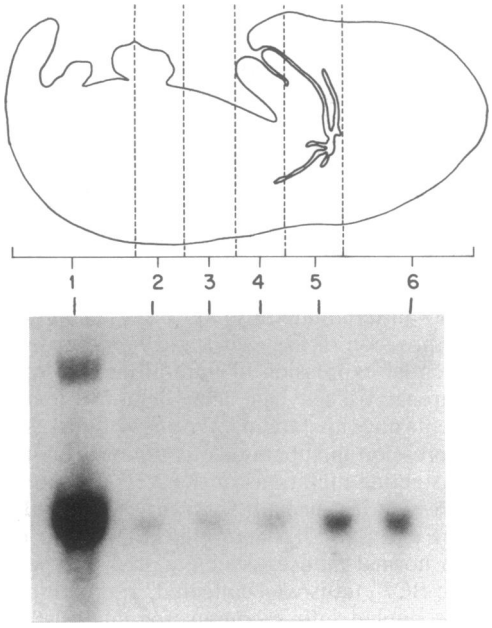

FIG. 4. Renin expression in a sectioned nontransgenic fetus. A normal 15.5-day postcoitus male BCF_2 fetus was frozen, and sections were pooled for RNA analysis. Segments 1 and 6 contained ≈3 mm of tissue, whereas segments 2–4 contained 1.5 mm of fetal tissue. Sixty micrograms of RNA was run on 1.5% agarose/formaldehyde gels and hybridized with a renin antisense RNA. The resultant signals were due to RNase A-resistant hybridization. Sections were saved every 100 μm for histological analysis. The major organs present in each segment are as follows: segment 1, gonads, kidneys, adrenal glands, bladder, and intestine; segment 2, liver, spleen, intestine, stomach, pancreas, and lung; segment 3, liver, heart, and lung; segment 4, heart, throat, sternum, forelimbs, and salivary glands; segment 5, oral cavity, salivary glands, and throat; segment 6, brain and neural tissues. All segments contained skin and underlying tissues.

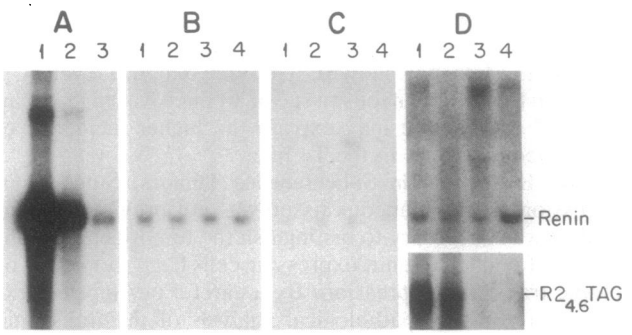

FIG. 5. Renin expression in normal subcutaneous tissue. RNA was extracted from eviscerated 15.5-day postcoitus fetuses and newborns and newborn skin, run on 1.5% agarose/formaldehyde gels, and hybridized with an antisense renin probe (*A–C* and *D Upper*) or an antisense T-antigen probe (*D Lower*). The resultant signals were due to RNase A-resistant hybridization. (*A*) Lanes 1–3 contain 1.0, 0.1, and 0.01 μg, respectively, of submandibular gland RNA from a male DBA/2J mouse. (*B*) Lane 1, 15.5-day postcoitus eviscerated DBA/2J fetus; lanes 2–4, independent 15.5-day eviscerated BCF_2 fetuses. (*C*) Lanes 1–4, independent skin samples from BCF_2 newborns. (*D*) Lanes 1 and 2, eviscerated transgenic newborns; lanes 3 and 4, eviscerated BCF_2 newborn (negative littermates). One hundred micrograms of RNA was loaded in each lane in *B–D*. Exposure time was 2 weeks. Blots were treated with RNase A to remove nonspecific hybridizing probe.

eviscerated 15.5-day postcoitus fetuses and newborns and newborn skin and was analyzed by Northern blot hybridization (Fig. 5). The results clearly demonstrated the presence of significant quantities of correctly processed renin mRNA from four independent eviscerated fetuses from DBA/2J (Fig. 5*B*, lane 1) and BCF_2 (Fig. 5*B*, lanes 2–4) mice. Renin mRNA was also detected in each of the independent eviscerated newborn carcasses (Fig. 5*D*), although the levels were more variable than in Fig. 5*B*. The RNA samples shown in Fig. 5*D* were derived from newborn mice of a cross between transgenic and nontransgenic mice. Accordingly, the transgene was transmitted to 50% of the offspring. Pups 1 and 2 tested positive for the transgene by dot blot analysis of their tail DNA and expressed the transgene in the eviscerated carcasses. Pups 3 and 4 were nontransgenic littermates. Finally, low but discernable renin mRNA was detected in four independent samples of newborn skin (Fig. 5*C*).

DISCUSSION

Evidence Identifying Subcutaneous and Subepidermal Tissues as Sites of Renin Expression. The presence of renin in many extrarenal tissues has been previously demonstrated by a combination of immunohistochemical and expression analysis (3–7). Recently, renin expression was detected by the polymerase chain reaction in several extrarenal adult tissues in which the relative abundance of renin message was found to be quite low (8). Here we report our experiments using transgenic mice containing an oncogenic reporter gene to identify previously unreported sites of renin expression. Our ability to express T antigen specifically in renin-expressing cells, and the proficiency of T antigen to transform some of these cells to a tumorigenic phenotype, allowed for the successful identification of renin-expressing cell populations in subcutaneous tissue.

Although extrarenal renin-expressing tumors in humans have been reported, they are rare and have often been described as ectopic (25–29). Our data strongly suggest that expression of the transgene in subcutaneous tissue is not ectopic in that (*i*) the endogenous renin gene (which codes for the circulating renin in these mice) is expressed in the T-antigen-induced subcutaneous tumors, (*ii*) these tumors can express a renin gene from an independent locus, and (*iii*) renin mRNA is present in eviscerated fetuses and newborns and newborn skin. Since multiple transgenic lines also demonstrated the same localization of T-antigen-containing cells (Fig. 1) and developed renin-expressing subcutaneous tumors, position effects on transgene expression are therefore ruled out as the sole cause of tumorigenesis in this tissue.

The location of the renin-expressing cells in subcutaneous tissue (as detected by T-antigen immunohistochemistry; Fig. 1) is consistent with a mesenchymal or primitive connective tissue origin. Although we were able to detect renin mRNA in eviscerated fetuses and newborns (normal and transgenic), we were unable to detect it in skin or subcutaneous or other underlying soft tissues from 6-day-old, 9-day-old, or adult mice by Northern blot (data not shown). It is unclear whether this decrease resulted from the progressive loss of renin-expressing cells after birth or was due to the dilution of these cells by the rapid growth of the surrounding tissue. Some pluripotent mesenchymal cells are believed to be present in mature connective tissues. Consequently, it is possible that a small population of these cells may have the potential to be recruited into a renin-expressing population upon proper stimulation. Recruitment of non-renin-expressing cells into a renin-expressing cell population has been demonstrated in the kidney (30). Tumorigenesis in our adult transgenic mice may have occurred as a result of stochastic events in such a population of cells. In light of this, it is interesting that renin-secreting human tumors derived from subcutaneous soft tissues have been observed (27).

Significance of Subcutaneous Renin Expression. An increasing body of evidence has led to the supposition that the local synthesis of renin and components of the RAS at several extrarenal sites may be physiologically important. The adrenal RAS has been proposed to assist in the regulation of electrolyte balance through controlling production of aldosterone (1, 31), and the intrarenal RAS has been proposed to have effects on the regulation of renal hemodynamics (32). In addition, the identification of components of the RAS in the ovary, ovarian follicular fluid, testicular Leydig cells, sperm, and seminal fluid (6, 10–12, 33, 34); the presence of A-II receptors in the testes and ovary (35); and reports consistent with a role for A-II in ovulation (36) have prompted the proposal that the RAS may participate in reproductive physiology.

Our identification of renin expression in subcutaneous soft tissues is particularly interesting in light of recent reports demonstrating a similar localization of A-II receptors (37, 38) and the identification of angiotensinogen mRNA in brown adipose tissue and mesentery (13). Although a functional relationship between local renin and A-II synthesis and its action on A-II receptor-containing cells has yet to be demonstrated, their localization strongly suggests that the RAS may play a role in this tissue. The possible functions may include a role in fetal development of subcutaneous and surrounding tissues. In this light, the close localization of the renin-expressing cells to the developing skeletal muscle is intriguing. Although a definitive role has yet to be demonstrated, support for a role in development stems from the following observations: (*i*) A-II may play a role in angiogenesis (39); (*ii*) the A-II receptor acts through the calcium/phospholipid pathway, which has been implicated as a route of mitogen action (40); (*iii*) subepidermal A-II receptors are present, which also rapidly decrease after birth (37); (*iv*) there appears to be a very specific population or lineage of renin-expressing cells in subcutaneous tissue during fetal development; and (*v*) renin mRNA was easily detected in eviscerated fetal and newborn carcasses but not the same adult tissues (Fig. 5). The RAS may also have multiple autocrine or paracrine effects on the subcutaneous and surrounding tissues of neonates and adults as evidenced by the provocative findings that A-II may elicit effects on inflammation (41), vascular permeability (42), vascular tone (43), and the release of norepinephrine from noradrenergic nerves (44). In this light, it is tempting to speculate that renin expression in subcutaneous tissues may play a role in the local production of A-II.

In summary, we have identified a site of renin synthesis in peripheral tissues of fetal and newborn mice and a potential source of renin-expressing cells in peripheral tissues of adults. The precise physiological relevance of renin produced at this site and at other extrarenal sites remains to be determined.

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