

STUDIES OF THE MURINE REN-2 PROMOTER
REGION IN TRANSGENIC MICE

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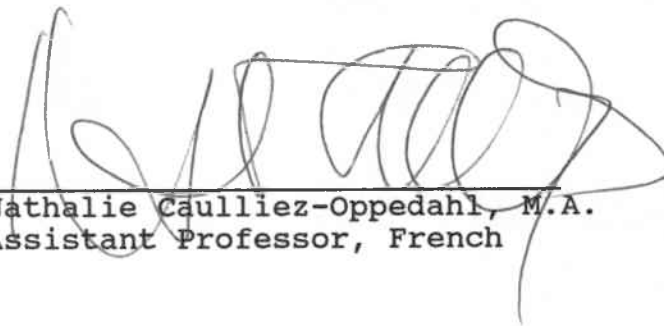
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ABSTRACT

In this study, a gene construct containing 4.6 Kb of Ren-2 5' flanking sequence fused to either the gene encoding β -galactosidase or to the T antigen was tested for its ability to confer a tissue specific expression profile in transgenic mice. Eight transgenic lines were analyzed for the presence of the transgene by Southern blotting techniques and by dot blot hybridization. Northern blot hybridization of total RNA isolated from tissues was performed to assay for the presence of transgene transcripts. No detectable transgene expression was observed in 5 transgenic lines. Testicular transgene expression was evident in 2 transgenic lines, and tissue specific expression was observed in 1 line. Application for the development of an assay for studying renin development during fetal development is discussed.

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INTRODUCTION

Renin is an aspartyl protease that participates in the regulation of blood pressure and electrolyte balance by catalyzing the reaction that converts angiotensinogen to angiotensin I (1). Renin is most often associated with the juxtaglomerular cells of the kidney. It is thought that the active form of renin is synthesized and stored in the juxtaglomerular cells (2). Many recent studies have shown that renin is also expressed in many extrarenal sites. These sites include the adrenal gland, testis, submandibular gland (SMG), and coagulating gland (3-5). The physiological function of renin at these sites is unknown.

Most mammals such as humans and rats carry only one gene that encodes renin (6,7). The mouse is particularly interesting because certain strains of mice carry an extra copy of the renin gene. The renin gene common to all strains of mice is designated as Ren-1 (8). The Ren-1 gene in strains such as C57BL/6 is known as Ren-1c, and in strains such as DBA is known as Ren-1d (1). Mice that contain the Ren-1d allele also carry a second tightly linked renin locus that is known as Ren-2 (9). Ren-2 seems to have arisen from a duplication at the renin locus about 3-10

million years ago (10,11). Ren-1 and Ren-2 have highly similar nucleotide sequence, and their transcripts have greater than 96% sequence identity (11).

The three genes are all expressed at the same level in the kidney, but exhibit unique expression patterns in extrarenal tissues (12,21). Since there is a high homology in the coding and flanking regions of the three genes, it was of interest to determine the DNA sequences that control the tissue specific expression of these genes. In these studies, transgenic mice were used to study the expression of the renin gene. Production of mice carrying the Ren-2 promoter region fused to a reporter gene allows for expression to be assayed in all tissue in vivo. This allows for the molecular dissection of the renin promoter region into elements responsible for tissue specific expression. The main goal of these studies was to test the ability of a gene construct containing 4.6 Kb of the Ren-2 5'flanking sequence fused to a gene encoding β -galactosidase to confer a tissue specific expression profile. An additional goal was to test the ability of a construct containing the SV40 T antigen to confer a tissue specific expression profile. Finally, the ultimate goal of these studies was to develop a sensitive assay for studying renin expression during fetal development.

LITERATURE REVIEW

Renin

Renin is most often associated with the renin-angiotensin-aldosterone system. The components of this system are essential for homeostatic control of blood pressure and fluid and electrolyte balance (1). Renin acts on alpha2-globulin angiotensinogen which is produced in the liver to produce the nonpressor decapeptide angiotensin I (13). Angiotensin I is then cleaved by the peptidyl dipeptidase angiotensin-converting enzyme to angiotensin II, which has pressor and aldosterone-stimulating activity (13).

The production of renin is a multiple stepped process. The sequence of events begins with the synthesis of preprorenin. Preprorenin is cleaved during translation to form prorenin. Prorenin is the immediate precursor of renin and is formed from the removal of a 23-amino-acid signal peptide from preprorenin (14). Preprorenin is synthesized and converted to prorenin in the juxtaglomerular cells and in many other tissues including the brain, peripheral vascular system, reproductive tissues, and in the adrenal medulla and cortex (15). Prorenin is also known as inactive renin. Active renin is formed from the removal of a 43-amino-acid prosegment from prorenin. The active renin is

then packaged for storage and release. It has been found that some prorenin is also released into the circulation (13). It is thought that measurements of prorenin plasma levels may serve as a marker for identifying patients with type I diabetes mellitus who are at risk of developing renal or retinal microangiopathy (16).

Renin genes present in the mouse

Mice are polymorphic for the number of Ren loci (12). All strains of inbred mice carry the Ren-1 structural gene (8). The Ren-1 allele present in some inbred strains (C57BL/6 and BALB/c) is designated Ren-1c (1). Other inbred strains (DBA/2 and SWR) carry the Ren-1d allele and a second tightly linked renin locus, whose allele is designated as Ren-2 (9). Ren-2 lies approximately 20 bases upstream of Ren-1d (6). Analysis of recombinant inbred strains has shown that Ren-1 and Ren-2 are genetically tightly linked and map near the Pep-3 locus on chromosome one (17). Ren-2 appears to have arisen as the result of a relatively recent duplication at the renin locus. It is thought that this duplication occurred three to ten million years ago (10,11). A model for the duplication has been proposed in which during meiosis, unequal crossing-over occurred between mispaired homologs of chromosome one (18). The chromosome bearing two renin genes was then maintained in a subset of murine subspecies. All three genes (Ren-1c, Ren-1d, and

Ren-2) are highly similar in nucleotide sequence within coding regions and in their genomic sequence organization (19). Comparison of nucleotide sequences has shown that the three genes share 97% similarity in their coding regions, and the similarity extends both 5' and 3' from the structural gene sequences (20). Although the genes are highly similar in sequence, they can be quantitatively distinguished by using a locus specific dideoxynucleotide (ddNTP) primer extension assay (1).

Expression of the renin genes

The three mouse renin genes are all expressed at the same level in the kidney, but exhibit unique expression patterns in extra renal sites (12,21). This expression profile is illustrated in Table 1. It is difficult to predict what gene will be expressed in a given tissue. All three genes may be expressed in one tissue (i.e. the kidney) but in another tissue only one or two of the genes may be expressed (i.e. the SMG, or coagulating gland) (22). Additionally in the fetal adrenal gland all three genes are expressed, but in the SMG only Ren-1c and Ren-2 are expressed and the Ren-2 gene is expressed 100 fold in excess of Ren-1c in the SMG. The mouse renin genes are similar in that all three genes are expressed in the kidney, initiate and terminate transcription at homologous positions, exhibit similar intron/exon structures, and are closely related in

Table 1. Tissue specific expression of the murine renin genes

Tissue	Ren-1c	Ren-1d	Ren-2
Kidney	+++	+++	+++
SMG	+	-	+++
Liver	-	-	-
Testes	+	++	+
Coagulating	+++	-	-
Fetal adrenal	+	+	+
Adult adrenal	-	+	+
Brain	-	-	-
Heart	-	-	-
Lung	-	-	-
Spleen	-	-	-
Muscle	-	-	-
Ovary	+	+	+
Fetal Subcutaneous Tissue	+	+	+

nucleotide sequence within coding regions (1). The Ren-1c and Ren-1d structural genes are presumed to be allelic based on a number of criteria. At the protein level, the products of the Ren-1c and Ren-1d are similar in enzymatic activity, thermostability, and immunological properties (23). Further, in contrast to Ren-2, the protein products of both Ren-1 alleles are glycosylated (3). Despite the high degree of similarity that exists between the murine Ren-1 genes, each gene exhibits its own characteristic allele-specific expression pattern (1). The tissue specific expression differences observed for the highly similar mouse renin genes most probably reflect changes that have occurred in the sequences associated with each gene, and these sequence changes affect expression in certain extra-renal tissues (i.e. sex accessory gland tissue, testis, adrenal gland, and SMG, but not in the kidney) (1). There are several identified insertion elements in the sequences flanking the coding regions of the mouse renin genes, the content of which varies among the three genes (24). Work is presently in progress using transgenic mice to examine the role of the insertion elements, and to identify the sequences associated with each gene that regulate allele and locus specific expression of the murine renin genes

Transgenic mice

Transgenic animals have incorporated into their genetic material genes that have been artificially transferred from an entirely different organism. The implanted genes can affect an animal's appearance by altering the color of its coat and an animal's health by making it prone to cancer (25). The production of transgenic mice involves the introduction of foreign genes into the mouse germ line. The resulting mice carry and express the foreign genes (26). Transgenic mice have a number of applications including studying control and tissue specificity of gene regulation, self-tolerance and autoimmunity studies, studies of oncogenes and cancer, the study of mammalian development, and in toxicology studies (25,27). The production of transgenic mice is a difficult and complicated process (28). Mice are treated so that they superovulate. Each mouse ovulates about twenty eggs. The mice are bred in order to produce fertilized eggs. The eggs are removed, and the DNA is injected into the male pronucleus with a super-fine needle. Varying copy numbers of the injected genes will integrate into the host chromosomal DNA. The eggs are surgically implanted into the oviducts of pseudopregnant foster mothers and allowed to develop to term (26,28). After the 19 to 20 days' gestation the DNA of the pups is tested for presence of the transgene. The procedure usually has an

efficiency rate of 20%, but it is common to have an efficiency rate of 10% or below. This procedure has many skill limitations and is very expensive. The microinjection technique can take many months to master, and the procedure requires about \$40,000 worth of special equipment (26). A new method for the production of transgenic mice has been suggested by Corrado Spadafora and his colleagues at the University of Rome. The group reported that they had produced transgenic mice by simply mixing mouse sperm with foreign DNA, and then using the DNA for in vitro fertilization (26). Eight independent researchers tried to repeat the potentially revolutionary technique. Each reported that they could not replicate Spadafora's results (26,29). So it seems that the only sure method of creating transgenic mice is to microinject the fertilized eggs.

Gene constructs

A diagram of the gene constructs used to produce the transgenic mice used in this study is shown in Fig. 1. All of the constructs used in this study are variations of a base construct that contains 4.6 KB of the Ren-2 5' flanking sequence fused to the E. coli lac Z gene. The Ren-2 5' flanking sequence is a potential regulatory element of the Ren-2 gene (21). The flanking sequence is duplicated from strains of mice carrying two renin genes, and exhibit high renin expression in the SMG. The base construct is known as

the BRLA construct. The BRLA construct is the first construct shown in Fig. 1. Transcription initiates within

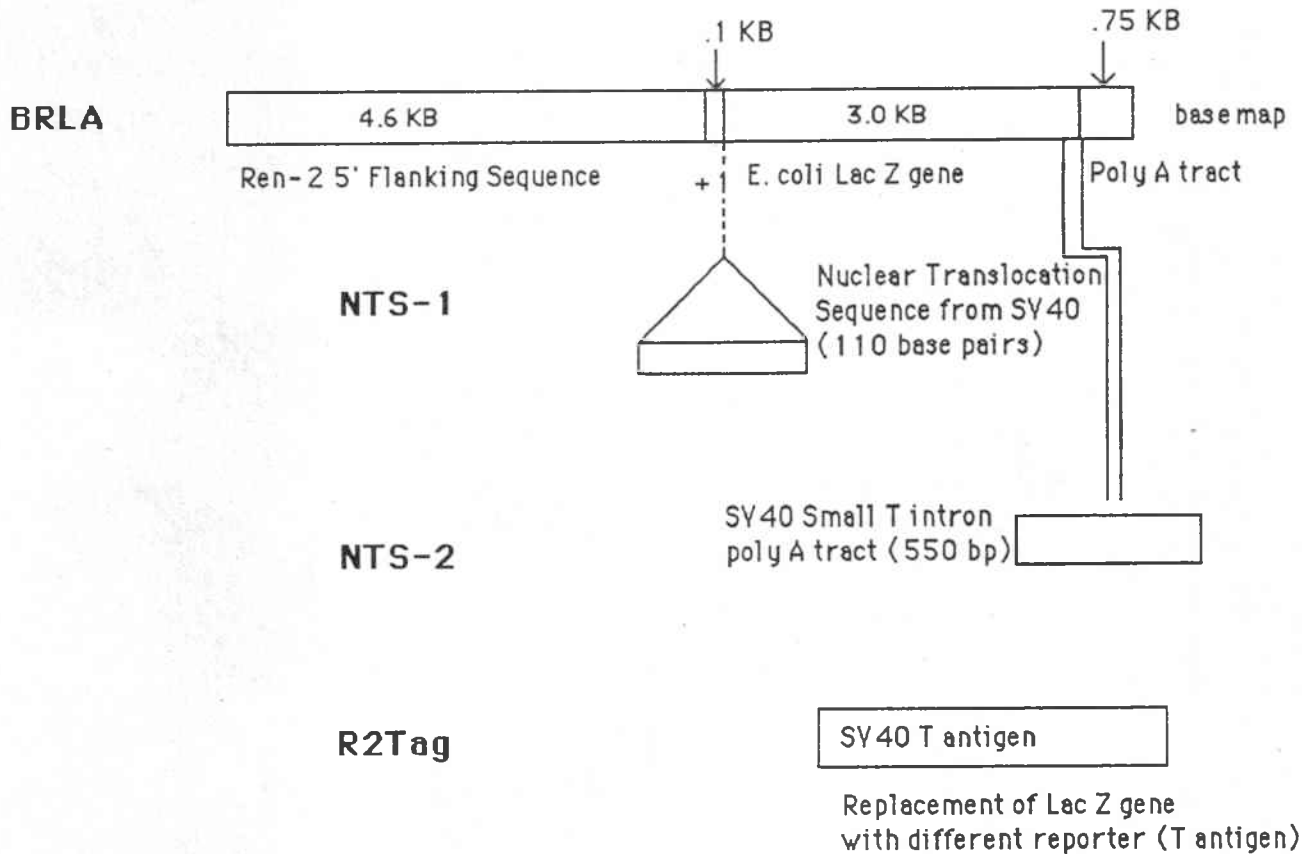


Fig. 1. Schematic representation of the constructs used in this study are shown. The base construct (BRLA) contains 4.6 KB of the Ren-2 5' flanking sequence fused to the E. coli lac Z gene. Transcription initiates within the Ren-2 5' flanking sequence. Transcription terminates within the poly A tract. NTS-1 is the same as BRLA except for the addition of a 110 BP sequence containing the nuclear translocation signal from SV40. NTS-2 is the same as NTS-1 except for the addition of an intron. R2 tag is the same as BRLA except that the E. coli lac Z gene had been replaced with the SV40 T antigen gene.

the Ren-2 flanking sequence and terminates within the poly A tract. The NTS-1 construct is the second construct shown in Fig. 1. The NTS-1 construct is the same as the BRLA construct except for the addition of a 110 base pair sequence containing the nuclear translocation signal from SV40. The nuclear translocation signal causes translocation of the β -gal protein to the nucleus where it may accumulate and therefore aid in its histochemical detection. The third construct in Fig. 1 is the NTS-2 construct. NTS-2 is the same as NTS-1 except for the addition of an intron. The presence of introns in transgenes has successfully been used to stabilize the transgenes' mRNA's and thus aid in the detection of transgene expression. The last construct in Fig. 1 is the R2 tag construct. The R2 tag construct is the same as the BRLA construct except that the E. coli lac Z gene has been replaced with the SV40 T antigen, which acts as a different reporter. These constructs consist of a putative Ren-2 flanking regulatory region fused to two different reporter genes. The reporters used in this study were β -gal and the SV40 T antigen. The reporter is used so the expression of the transgene is easily detected. This study focused on the β -gal gene, because it is very easy to histochemically detect the expression of the gene.

Renin expression during fetal development

Primary expression of renin occurs in the fetal kidney

and adrenal gland and can be detected 14.5 days post coitum (pc) (30). The development profile of renal renin expression is identical in strains of mice containing either the single renin gene or containing the duplicated locus. In the kidneys, this is consistent with the notion that all three renin genes are expressed at equivalent levels in adult organs (12). Surprisingly, expression of the Ren-1c gene, which is not detected in the adult adrenal gland is evident in the fetal adrenal gland between 14.5 and 16.5 days pc (30). The expression pattern of adrenal gland Ren-1c differs from Ren-1d and Ren-2 in that its expression decreases to undetectable levels between 16.5 days pc and birth while adrenal gland expression in strains of mice containing two renin loci continued after birth into adulthood (30). The strain specific expression differences observed in the adult adrenal gland appear to be regulated by sequences associated in cis with each allele (1). The lack of detectable Ren-1c expression in the adult adrenal gland appears to reflect a developmental down regulation of the steady state level of renin transcripts before birth (30). The cis-acting sequences which cause differential transcription in late gestational adrenal glands is in the process of being identified. Renin expression during fetal development may also serve as a marker for renal vascular development. Kidney renin transcripts are first detected at 14.5 days pc in the newly developing arteries. As the renal

arterial tree develops, renin mRNA containing cells are progressively localized to more distal blood vessels and finally to the specialized cells of the afferent arteriole (juxtaglomerular cells) (30). The transient localization of renin mRNA in cells of the fetal intrarenal arteries is consistent with the notion that renin may be a useful marker for the developing renal vasculature.

MATERIALS AND METHODS

Production of transgenic mice

The transgene constructs were produced by cloning the 4.6 KB of Ren-2 5' flanking sequence upstream to either the gene encoding β -galactosidase or the SV40 T antigen structural gene. For microinjection, the transgene segment was excised, purified by agarose gel electrophoresis and prepared as previously described (31). Transgenic mice were produced by standard methods with approximately 100 copies of the transgene injected into the male pronucleus of fertilized one-celled embryos. Methods for the production of transgenic mice have been previously described (28).

Identification of transgenic founder lines

Not all of the resultant pups will be transgenic; consequently when the suspected transgenic mice were born, the true positives had to be identified so that a line of mice carrying the transgene could be produced. DNA was isolated from the pups through tail biopsies. The DNA was purified, and the concentration determined by O.D. at 260nm. A restriction digest was done to separate the DNA into fragments. Each DNA restriction digest contained a total volume of 50ul: 10ug of purified DNA, 5ul of 10X buffer, 2ul each of the endonucleases Eco R1 and Bam H1, and the remainder water. The reagents were added together and

then incubated at 37° for 4 to 5 hr.

After the incubation period, a Southern blot was done. The DNA fragments were separated by agarose gel electrophoresis. A 0.8% gel was used to separate the DNA's. Agarose (1.6g) was added to 200ml of 1X gel buffer. The mixture was heated to 100°, and the agarose was allowed to dissolve in the buffer. The gel mixture was then cooled to 65°. The gel mixture was then poured into a horizontal gel electrophoresis box and left for 45 min so that it could harden. The gel was then covered with 1X stock gel buffer, and the DNA samples were loaded into the gel wells. Before the DNA samples were loaded, 5ul of dye was added to each sample. The gel was then run at 30V for 10-12 hr.

A pump is placed in the gel box so that the gel buffer is circulated and does not become stagnant. After the allotted time, the gel was removed from the box and placed in 1l of buffer solution that contained 100ul of ethidium bromide (EtBr). The EtBr serves to stain the gel. The gel was stirred in the presence of the EtBr for 15 min. The EtBr solution was then removed, and the gel was rinsed with distilled water for 10 min. It was then possible to visualize the DNA by photographic methods. The DNA in the gel was then placed in a denaturing solution and allowed to sit for 30 min. The denaturing solution consisted of 1.5M NaCl, 0.5M NaOH, and 0.25M EDTA. After the 30 min the gel was placed in a neutralizing solution for 30 min. The

neutralizing buffer consisted of 3M NaCl, and 0.5M Tris base. The solution was adjusted with HCl to a pH of 7.0.

The DNA was then transferred from the gel to a suitable medium. Nitrocellulose is often the medium of choice. To do the transfer, the gel was first measured. Four pieces of blotting paper were then measured. Two of the pieces were 4 cm larger than the gel and 2 pieces were 2 cm larger than the gel. A piece of nitrocellulose that was 1 cm larger than the gel was also measured. The transfer box consisted of a plastic box that contained sponges that were approximately 10 cm larger than the gel. The sponges were saturated with 10X SSC and SSC was also placed in the bottom of the transfer box. The 2 large pieces of blotting paper were placed directly on the sponges. The blotting paper was allowed to soak up some of the SSC. The gel was then placed well side down on the blotting paper. The gel was then framed in parafilm to facilitate the transfer. The nitrocellulose was then placed on top of the gel. Care was taken to ensure that no air bubbles existed between the gel and the nitrocellulose. The smaller pieces of blotting paper were then placed on top of the nitrocellulose. Stacks of paper toweling were then placed on top of the blotting paper. A weight was added to the toweling and the transfer was allowed to sit overnight.

After the transfer was complete, the nitrocellulose was allowed to air dry and then was placed in the vacuum dryer

for 2 hr. The nitrocellulose was then prehybridized and hybridized with a probe common to the endogenous renin gene and the transgene. In order to do this the probe was first labeled with ^{32}P . The probe was known as the R1SST probe. To label the probe, 5ul of OLB, 1ul of BSA, 13ul of water, 2.5ul of ^{32}P , 3ul of the probe (heated at 90° for several minutes), and 0.5ul of klenome were added together and then left overnight. A column was run to separate the large radiolabeled fragments from the smaller unusable fragments. The column was stacked with beads and covered with 1XTE buffer. The probe was then added to the top of the column. The radioactivity was monitored by a geiger counter. After a short period 2 peaks of radioactivity appeared in the column. The usable probe was located in the first peak. The first peak was collected and stored for future use.

The nitrocellulose blot was then prehybridized for 3 hr in a solution that contained 1X SSC, 0.1% SDS, 50ug/ml salmon sperm DNA, and 5X Denhardts solution. After the 3 hr, the solution was released and was replaced with the hybridization solution which contained 1X SSC, 0.1% SDS, 50ug/ul salmon sperm DNA, 5X Denhardts solution, and 10% Dextran solution. The labeled probe was also added to the hybridization solution. The probe was first boiled for 5 min. After the probe was added, the blot was left in a shaking water bath overnight. The hybridization solution was then released and the blot was placed in a container

containing a wash solution of 1X SSC, and 0.1% SDS. A surface wash was first done and then the blot was placed in the water bath for 20 min. After the 20 min, 3 more 30 minute washes were done. The blot was then dried and wrapped in plastic wrap. The blot was then covered with film and allowed to expose for several days. After the required exposure time, the blot was developed and the transgenic founder mice could easily be identified.

Identification of transgenic offspring

After the positive founder mice were established, they were bred with nontransgenic mice. Since the founder mice carry the transgene in a single chromosome, under ideal circumstances only 50% of the offspring are transgenic. Since one is only interested in the transgenic animals, the offspring of these breedings must be tested for the presence of the transgene. This process began with the isolation of DNA from the pups. DNA was obtained from tail biopsies. One half inch was cut from the end of each pup's tail. The piece was then cut into 5 or 6 smaller pieces. Stock (700ul) tail buffer, and 35ul of proteinase K was added to the tail pieces. The mixture was rotated at 55° overnight. After the incubation period, 1ul of RNase was added to each sample. This was then allowed to rotate for 30 min at 37°. To each sample, 700ul of PCI was added. The samples were then allowed to rotate for 30 min. The tubes were then

centrifuged. After centrifugation 2 layers were present. The top layer was retained, and the bottom layer was discarded. PCI (700ul) was again added to each sample. The samples were rotated for 30 more min. The samples were again centrifuged, and the layers were separated. The tubes were filled with 700ul of CIA. The samples were allowed to rotate for 30 min. The samples were centrifuged, and the layers were separated. The tubes were filled with isopropanol and allowed to sit for several minutes. A glass rod was then twirled in each sample to collect the DNA. The DNA sticks to the glass rod and the strands wrap around the rod as it is twirled. The spooled DNA was washed first in 70% and then in 100% ethyl alcohol. The DNA was allowed to dry for 30 min. After the 30 min, the DNA was resuspended in 200ul of water and left overnight. The concentration of the DNA was determined by O.D. at 260nm.

To test the DNA for the presence of the transgene, dot blot hybridization was performed. For each sample, a 50ul solution was prepared that contained 2ug of DNA, 25ul of 0.8M NaOH, and the balance was water. The samples were then incubated at 65° for 10 min. After the incubation, 50ul of 2M ammonium acetate was added. The solution was then vortexed and put on ice. At this point the dot blot manifold was prepared. A piece of nitrocellulose was placed in the manifold. A slight vacuum was started, and 100ul of 1M ammonium acetate was placed in each dot. The samples

were then loaded into the dot blotter. An additional 100ul of 1M ammonium acetate was added to each dot. The blot was then removed from the manifold and washed in 4X SSC. The blot then air dries for 30 min and is vacuum baked for 2 hr.

The blot was then hybridized with a probe specific for the transgene and not the endogenous gene. This probe was known as the β -gal probe. Before the probe was used, it was first labeled. To label the probe 5ul of OLB, 1ul of BSA, 13ul of water, 2.5ul of ^{32}P , 3ul of the probe (heated at 90° for several minutes), and 0.5ul of klenome were added together and let sit for several hours. After the allotted time, the probe was run through a column so that the usable fragments were obtained. Before the probe was added to the blot, the blot was first prehybridized. The prehybridization solution consisted of 1X SSC, 0.1% SDS, 50ug salmon sperm DNA, and 5X Denhardts solution. The prehybridization solution was added to the blot and left in a water bath for 2 hr. The solution was then discarded, and a hybridization solution was added which consisted of 1X SSC, 0.1% SDS, 50ug/ml sperm salmon DNA, 5% Denhardts, and 10% Dextran. The probe was also added after it had been boiled in water for 5 min. The blot was then placed in a water bath and left overnight. The liquid was then released and the blot was washed with a solution consisting of 1X SSC and 0.1% SDS. Three half-hour washes were done with this solution and then a final wash was done with a solution

containing 0.1% SSC and 0.1% SDS. The blot was then allowed to dry. After drying, the blot was placed on film and allowed to exposed overnight. The film was then developed and the transgenic offspring were identified.

Localization of RNA from tissues

After the positive animals were identified, the animals were dissected. The animals were euthanized by means of carbon dioxide. Each animal was then placed on its back in a dissecting tray. Rubbing alcohol was placed on the abdomen to smooth the fur. The skin of the abdomen was lifted up, and a small cut was made in the fur. The fur was then pulled away to expose the muscle layer. The muscle was cut to expose the internal organs. The internal organs were then taken out in the following order: adrenal glands, kidneys, testes, seminal vesicles, spleen, liver, heart, lungs, SMG, and finally the brain. Tissues were then selected for RNA extraction. In theses studies, RNA was extracted from the kidney, liver, SMG, and testis. The kidney, SMG, and testis are renin positive tissues while the liver is a renin negative tissue. During RNA extraction it is important to wear gloves at all times as substances present on the hands can destroy RNA. GITC solution is needed for the RNA extraction. To 50ml of GITC 350ul of 2-mercaptoethanol and 10 drops from a pasteur pipette of antifoam emulsion were added. The solution was then mixed

and put on ice. Two milliliters of GITC solution was added to a tube that contained 1 of the tissue samples. The tissue was then homogenized in the liquid solution. After the tissue was completely homogenized, an additional 6 ml of GITC solution was added to the tube, and the tube was placed on ice. The RNA was then pelleted out of the solution by means of differential centrifugation.

The sample was loaded into 14X89 ultra-clear centrifuge tubes, and a SW-41 rotor was used. Three milliliters of 5.7M CsCl solution was added to each centrifuge tube. The liquid samples were then layered on top of the CsCl solution. It is important at this step to make sure that no mixing occurs between the sample and the CsCl. The tubes were then filled to the top with DEPC water. The tubes were then balanced and placed in the rotor. The tubes were then centrifuged for 18 hr at 15° and at a speed of 35,000 RPM. After the 18 hr, the centrifuge was stopped and the tubes released. Using a mild vacuum, the liquid was aspirated out of the tube. The RNA pellet is located on the bottom of the tube. The tube was turned upside down and allowed to dry for 1 min.

The RNA pellet was resuspended in 400ul of DEPC water and placed on ice for 30 min. The sample was placed in a tube that contained 50ul of 3M sodium acetate, pH 5.0. The tubes were filled with ethanol, vortexed, and stored at -20° overnight. After the elapsed time, the samples were removed

from the freezer and centrifuged for 10 min at 10°. The liquid was poured off, and the tubes were allowed to dry for 1 min. Seventy percent ethanol was placed in the tubes and the tubes were centrifuged for 5 min. The liquid was released and the tubes were allowed to dry for one minute. The tubes were filled with 70% ethanol and centrifuged for 5 more min. The liquid was poured off and the tubes were allowed to dry for several minutes. The tubes were placed in the vacuum dryer for 15 min. The RNA was resuspended in 400ul of DEPC water and placed on ice for 30 min. The concentration of the RNA could then be determined by O.D. at 260nm.

Identification of renin expressing tissues

Northern blot hybridization was performed to identify expressing tissues. To do Northern blots, a 10X MOPS buffer was first prepared. To make the buffer, 800ml of water was added to 41.8g of MOPS. The pH was then adjusted to 7.0 with NaOH (3g). To this, 16.6ml of 3M DEPC treated sodium acetate was added. The next component added was 20.0 ml of 0.5M DEPC treated EDTA, pH8. The solution was then brought to the final volume of 1l by adding DEPC treated water.

The first step of the Northern blot involves centrifuging the samples for 10 min. After the 10 min, 2 ethanol washes were done and the samples were placed in a speed vac for 15 min. The samples were then resuspended in

22ul of DEPC water and put on ice for 15 min. A 1.5% agarose gel was then prepared. Three grams of agarose was placed in 175ml of water. The agarose was dissolved in the water and heated to 100°. The gel was then allowed to cool to 65°. When the solution reached 65°, 20ml of 10X MOPS buffer and 6ml of 30% formaldehyde were added. The gel mixture was then poured into a horizontal gel box and allowed to harden. A solution was then prepared which was added to the samples. The solution consisted of 150ul of 10X MOPS, 262ul of 30% formaldehyde, and 750ul of formalin. Seventy-eight microliters of the solution was added to each sample. The tube was then vortexed and put in a hot block for 15 min at 50°. After the gel hardened, it was covered with 2l of 1X MOPS buffer. After the samples had been heated, 4ul of loading dye was added to each sample. The samples were then loaded into the gel wells and the gel was run for 4 hr at 90V.

After the gel had run, the power was turned off, and the gel was placed in 1l of water for 10 min. The gel is then placed in 10X SSC and shaken for 45 min. A transfer blot similar to the one used in a Southern blot was then set up. The transfer medium was nitrocellulose, and the transfer was left overnight. After the transfer, the blot was air dried for 30 min and then vacuum dried for 2 hr. The blots were then placed in a prehybridization solution that consisted of 50% formalin, 10mm sodium phosphate, 10X

Denhardt's, 300ug/ul salmon sperm DNA, 4X SSC, and water. The blot was placed in the prehybridization solution for 2 hr.

One of two identical blots was probed for transgene expression and the other blot was probed for the endogenous renin gene. These probes were first labeled with radioactive ^{32}P -GTP. To do this the following components were added together: 1.5ul adenine, 1.5ul cytosine, 1.5ul guanine, 1.5ul uracil, 3ul 5X buffer, 1ul DTT, 0.5ul RNasin, 2ul DNA, 5ul ^{32}P -GTP, and 0.5ul SP6. The probe was then heated at 37° for 30 min. SP6 (0.5ul) was again added and the tubes were left to incubate for an additional 30 min. The probes were then run through columns so that the radiolabeled fragments were recovered. The prehybridization solution was released from the blot and the hybridization solution along with the labeled probe was added to the blot. This was allowed to shake in a 65° water bath overnight. The blot was then washed in a solution that contained 0.1% SDS and 0.1X SSC. Three 20 min washes were done, and then the blot was dried and wrapped in plastic wrap. The blot was then placed on film and allowed to expose for several days. The film was then developed and the expressing tissues were identified.

X-gal staining of transgenic embryos

In order to stain embryos a fix, wash, and stain

solution were prepared. The fix solution consisted of 4.0ml 10% glutaraldehyde, 0.4ml $MgCl_2$, 10ml 0.1M EGTA, 20ml 1M phosphate, and 165.6ml of water. The wash solution consisted of 100ml 1M phosphate, 10ml NP40, 10ml Na deoxycholate, 2ml $MgCl_2$, and 878ml of water. The stain solution consisted of 2ml phosphate, 0.2ml Tris, 0.04ml $MgCl_2$, 0.5ml X-gal, 1.34 ml K-Ferri/Ferro-CN, 0.2ml NP40, 0.2ml Na deoxycholate, and 15.7 ml of water. Embryos were allowed to develop to desired size. The mother was then euthanized and the embryos and placentas were removed. The embryos were placed in the fixing solution for 30 min. The fix solution was removed, and the washing solution was added. The embryos were washed 3 times. Each wash lasted 30 min. After the final wash, the washing solution was removed and the staining solution was added. The embryos were then left to incubate at 37° overnight. After the elapsed time, the embryos were examined and staining patterns were noted.

RESULTS AND DISCUSSION

Establishment of founder lines

Six mice were found to carry the transgene and were capable of becoming founder mice. Fig. 2 illustrates a typical Southern blot identifying positive founder mice. Eight suspect founder mice were tested for the presence of the transgene. Since the dissected DNA from the endogenous renin gene and the transgene are of different sizes, they migrate differently in agarose gels. Thus, if the transgene is present, 2 bands will form on the blot. The top band on the blot is the endogenous renin gene, and the bottom band is the transgene. From the presence of the 2 bands, animals 1869/4, 1872/4, 1872/2, 1874/3, 1874/2, and 1874/1 were determined to be positive transgenic founder mice. Of these positive founder mice 4 contained the BRLA construct and 4 contained the NTS-1 construct. It can also be seen in Fig 2 that the transgene band shows varying degrees of intensity. This shows that different copy numbers of the construct are integrated into the DNA. The founder mice were then bred to nontransgenic mice to produce a line of mice carrying the transgene.

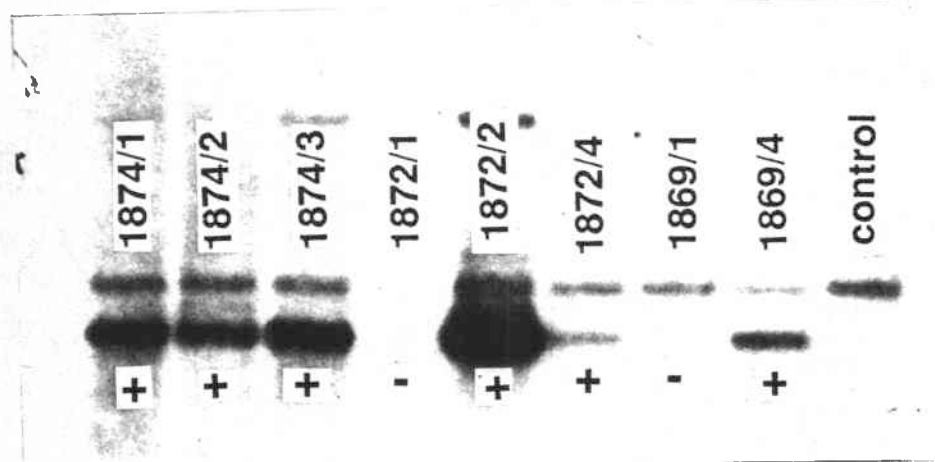


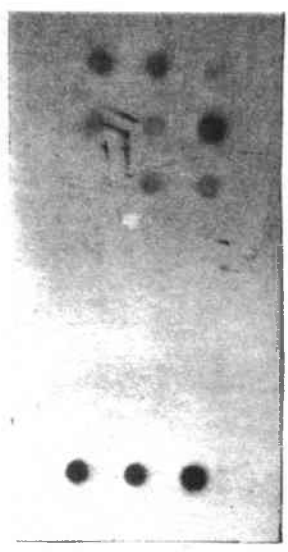
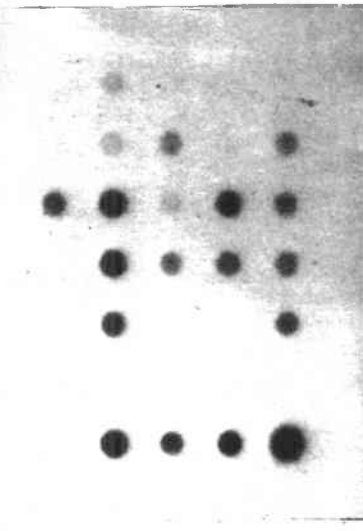
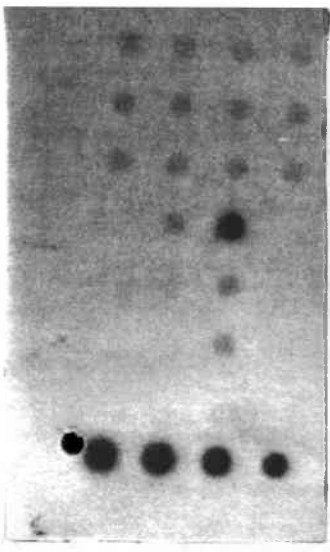
Fig. 2. A representative Southern blot identifying positive transgenic founder mice. DNA isolated from tail biopsies was digested with Eco R1 + BAM H1, separated by electrophoresis, and hybridized with a probe common to the endogenous renin gene and the transgene. +, positive transgenic founder; -, negative littermates. top band, endogenous renin gene; bottom band, transgene.

Identification of transgenic offspring

The identification of transgenic offspring was done by dot blot hybridization. Fig. 3 illustrates typical dot blots of the prospective transgenic offspring. The bottom row of dots are control DNA from the transgenic founder mice that were previously identified. A positive animal has a dot that is at least as intense as the founder dot. Out of 50 animals tested, 11 transgenic mice were identified. In some cases transmission of the transgene is less than 50%. This often occurs because the DNA integrates late, and the founder is known as a mosaic. This means that less than 50% of the founder's gametes contain the transgene.

Identification of renin expressing tissues

After the positive animals were identified, they were dissected, and RNA was extracted from the kidney, liver, SMG, and testis. Northern blot hybridization was then performed to identify expressing tissues. Fig. 4 illustrates representative Northern blots of RNA isolated from the mentioned tissues. Tissues were probed with a probe specific for the endogenous renin gene and also probed with a probe specific for the transgene. Three mice were placed on each blot. There were always two positive mice and one nontransgenic control. It was found that renin is expressed in the kidney, SMG, and testis but not in the liver which is the typical pattern of renin expression. The



-	-	-	-
-	-	-	-
-	-	-	-
	-	+	
		-	
		-	
Control	Control	Control	Control

-	-	-	-	-
-	-	+	-	-
+	+	-	+	-
	+	+	+	-
	-			-
Control	Control	Control	Control	Control

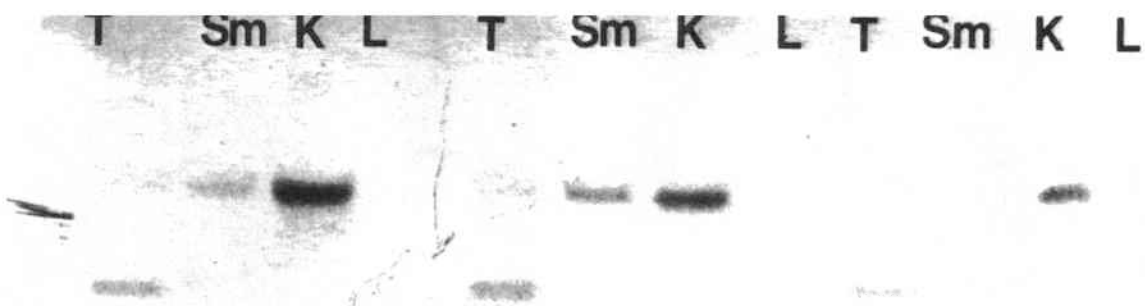
+	+	-
-	-	+
	-	-
Control	Control	Control

2221/1	2222/1	2223/1	2224/1
2221/2	2222/2	2223/2	2224/2
2221/3	2222/3	2223/3	2224/3
	2222/4	2223/4	
		2223/5	
		2223/6	
1874/1	1874/1	1874/2	1872/4

2173/1	2174/1	2175/1	2176/1	2177/1
2173/2	2174/2	2175/2	2176/2	2177/2
2173/3	2174/3	2175/3	2176/4	2177/3
	2174/4	2175/4	2176/5	2177/4
	2174/5			2177/5
2015/1	2020/2	2015/3	2022/1	2017/1

2225/1	2226/1	2227/1
2225/2	2226/2	2227/2
	2226/3	2227/3
2015/3	2015/1	2020/2

Fig. 3. Representative dot blots identifying positive transgenic offspring. Three micrograms of crude tail DNA was denatured and applied to nitrocellulose using a dot blot manifold. Blots were hybridized with a probe specific for the transgene. Control DNA from each of the transgenic founders is present in the bottom row. Animal numbers and identification of transgenic mice is summarized below each dot.



● Renin

T Sm K L T Sm K L T Sm K L



B-gal

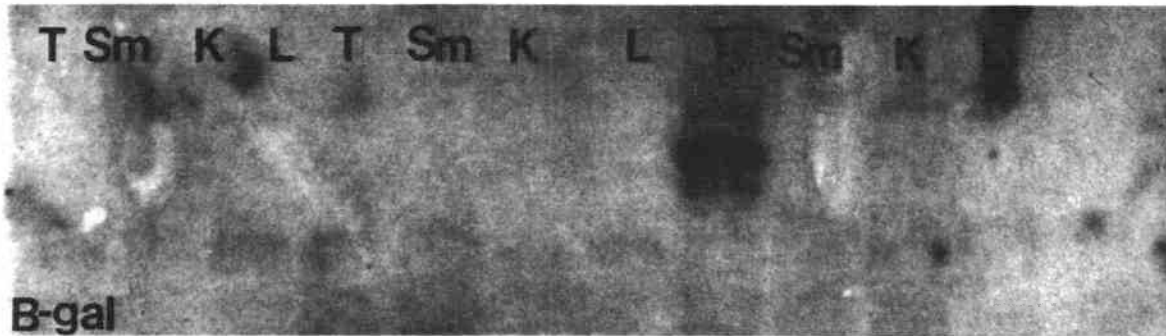


Fig. 4. Representative Northern blots of RNA isolated from Kidney (K), Submandibular gland (Sm), Testis (T), and Liver (L), from two transgenic mice and one nontransgenic littermate. Top panel: endogenous renin gene probe. Middle panel: transgene probe. Bottom panel: long exposure of middle panel.

transgene was expressed only in the testis of one transgenic mouse. It was not seen in any other tissues. No β -gal expression was observed in any tissues from the 3 BRLA lines examined or from 2 of 4 NTS-1 lines examined. Only 2 NTS-1 lines contained this testicular expression.

Renin expression of mice carrying the R2 tag

Northern blot analysis was performed on the SMG, testis, spleen, kidney, liver, heart, lung, muscle, and brain from transgenic mice carrying the R2 tag. Fig. 5 illustrates representative Northern blots from these tissues. The top blot was probed for renin expression, and the bottom blot was probed for the T antigen. In the top blot renin expression was evident in the SMG, kidney, and testis. In the bottom blot the T antigen expression was also evident in the SMG, kidney, and testis, and was mainly restricted to these sites.

Table 2 is a summary of the animals tested in this study. Animals are listed with respect to the transgenic line they were derived and the transgene construct that they contain. Transgenic mice are identified either with a + or - and the animals that were dissected for RNA analysis are indicated.

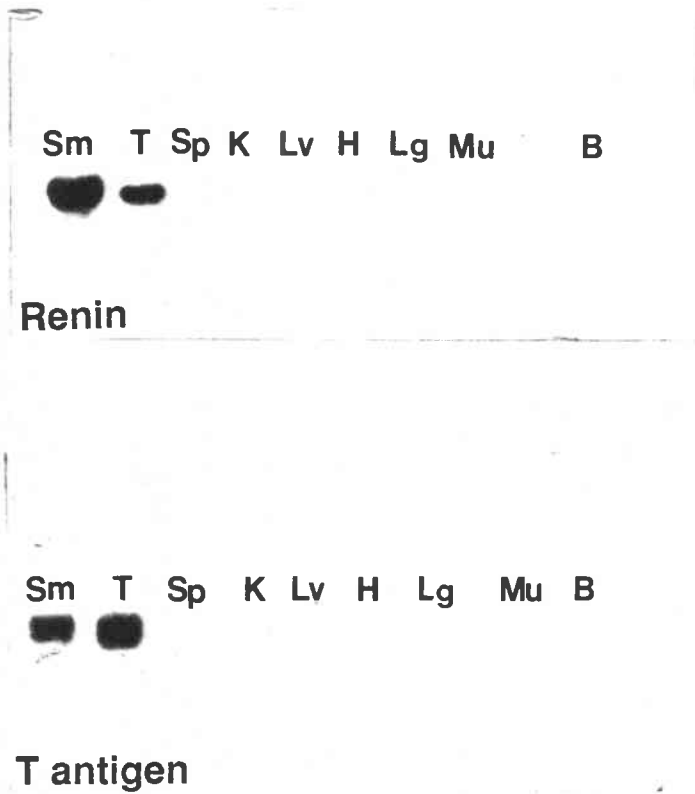


Fig. 5. Northern blot analysis of R2 tag transgenic mice. Whole tissue RNA from Submandibular gland (Sm), Testis (T), Spleen (Sp), Kidney (K), Liver (L), Heart (H), Lung (Lg), Muscle (M), and Brain (B) were prepared and probed for renin and the T antigen as indicated.

Table 2. Summary of animals tested in this study.

Animal	Line	Construct	Transgenic	Dissected RNA Northern
2173/1	2015/1	NTS	-	-
2173/2	"	"	-	-
2173/3	"	"	+	+
2174/1	2020/2	"	-	+
2174/2	"	"	-	-
2174/3	"	"	+	-
2174/4	"	"	+	+
2174/5	"	"	-	-
2175/1	2015/3	"	-	-
2175/2	"	"	+	-
2175/3	"	"	-	-
2175/4	"	"	+	+
2176/1	2022/1	"	-	-
2176/2	"	"	-	-
2176/4	"	"	+	+
2176/5	"	"	+	+
2177/1	2017/1	"	-	-
2177/2	"	"	-	-
2177/3	"	"	-	-
2177/4	"	"	-	-
2177/5	"	"	-	-
2178/1	1872/1	BRLA	-	-
2178/2	"	"	-	-
2178/3	"	"	+	+
2179/1	1874/1	"	-	+
2179/2	"	"	-	-

Table 2. Continued

Animal	Line	Construct	Transgenic	Dissected RNA Northern
2179/3	"	"	-	-
2179/4	1874/1	BRLA	-	-
2179/5	"	"	-	-
2180/1	1872/4	"	-	+
2180/2	"	"	-	-
2180/3	"	"	+	-
2180/4	"	"	+	+
2181/1	1874/2	"	-	-
2182/1	1869/4	"	-	-
2182/2	"	"	-	-
2182/3	"	"	-	-
2182/4	"	"	-	-
2221/1	1874/1	"	-	-
2221/2	"	"	-	-
2221/3	"	"	-	-
2222/1	"	"	-	-
2222/2	"	"	-	-
2222/3	"	"	-	-
2222/4	"	"	-	-
2223/1	1874/2	"	-	-
2223/2	"	"	-	-
2223/3	"	"	-	-
2223/4	"	"	+	+
2223/5	"	"	-	-
2223/6	"	"	-	-

Although the β -gal fusion transgenes were not expressed in the SMG and kidney', the data obtained through the use of the T antigen transgene suggests that the 4.6 KB of the Ren-2 5' flanking sequence can confer a tissue specific expression profile upon an exogenous reporter gene. It is possible that sequence elements present in the T antigen transgene but absent from the β -gal transgene, such as introns, may stabilize the mRNA and allow detectable expression in tissues other than the testes. Such a construct, similar to NTS-1 but containing an intron is now in the process of being tested in transgenic mice. This new construct is known as NTS-2. It is possible that slight modifications of the constructs may allow one to direct expression of β -gal to the correct spectrum of tissues.

Further directions: Development of an assay for studying renin expression during fetal development

Fig. 6 illustrates the results of an attempt to develop an assay for studying renin expression during fetal development. BRLA embryos were recovered from timed pregnant females at 13, 14, and 15 days of gestation. Embryos were fixed in situ and then stained overnight in a histochemical reagent specific for β -galactosidase. Three

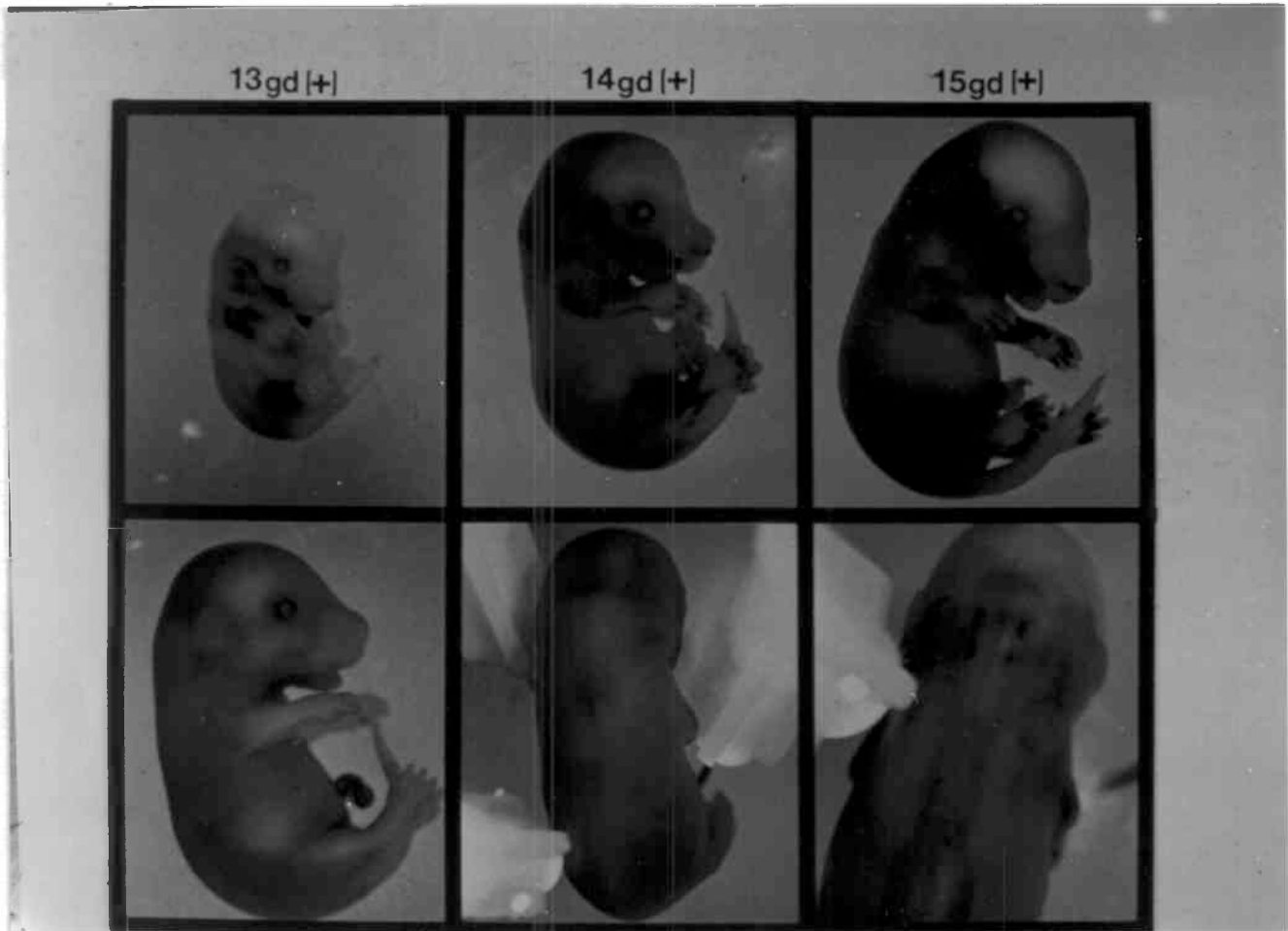


Fig. 6. X-gal staining of transgenic embryos. Three positive transgenics are shown along the top panel and the lower left hand panels. Negative littermates are shown in the lower right hand panels. This pattern of staining on the developing limbs is unique to this line of transgenic mice. These data are included to illustrate the utility of the approach of using a highly sensitive reporter for studying developmental expression of a certain gene.

positive transgenics are shown along the top panel and the lower right panel. Negative littermates are shown in the lower left hand panels. This pattern of staining on the developing limbs is unique to this line of transgenic mice. There was no staining evident in the fetal adrenal gland or kidney, the normal sites of renin expression during fetal development, suggesting that the site of transgene insertion is having an effect on expression of the transgene. These data are included to illustrate the utility of the approach of using a highly sensitive reporter for studying developmental expression of a certain gene. Work is underway to test the other transgenic lines used in this study as well as the new NTS-2 transgenics which are now available. The ultimate goal of this work is to study expression of renin early in fetal development when its expression is too difficult to detect by standard techniques.

Summary

Transgenic mice were obtained using three different constructs. These were the BRLA construct, the NTS-1 construct, and the T antigen construct. Transgenic founders were identified by Southern blot analysis. Eleven transgenic mice were identified by dot blot analysis out of a total of 51 animals tested. Mice representing 4 NTS-1

lines, 3 BRLA lines, and 1 R2 tag were analyzed by northern blot hybridization. Endogenous renin transcripts were identified in the kidney, testis, and submandibular gland. Renin transcripts were not identified in the liver, spleen, heart, lung, muscle, or brain. There was no detectable transgene expression in any tissue of 3 BRLA lines or 2 of 4 NTS lines. β -gal transcripts were evident at high levels in the testis in 2 NTS-1 lines. R2 tag transcripts were evident in the SMG, testis, kidney, and ovary (not shown). Expression was also seen in the brain which is a reported site of renin expression. These results suggest that the 4.6 KB of Ren-2 5' flanking sequences can confer a tissue specific expression profile on a reporter gene. Future directions include utilizing β -gal as a reporter for renin expression by applying the histochemical assay during fetal development.

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