The human plasminogen activator inhibitor type I gene promoter targets to kidney

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Emert, Martin P., Christine M. Sorenson, David P. Basile, Joseph G. Rogers, Marc R. Hammerman, and Joseph J. Billadello. The human plasminogen activator inhibitor type I gene promoter targets to kidney. Am. J. Physiol. 274 (Renal Physiol. 43): F405–F412, 1998.—The plasminogen activator inhibitor type 1 (PAI-1) gene encodes the physiological inhibitor of tissue-type and urokinase-type plasminogen activators and is induced by cytokines such as transforming growth factor-β (TGF-β). Studies have identified DNA sequence elements within the first 1.3 kb of the 5′-upstream DNA that mediate cytokine responsiveness in transfected cells in vitro. However, the DNA sequences that mediate PAI-1 expression in vivo have not yet been delineated. To define these regulatory sequences, we generated transgenic mice that expressed a hybrid gene comprising upstream sequences target transgene expression to kidney, primarily to adult and was seen primarily in proximal tubule cells of the outer medulla. Transgene expression detected in two independent lines was observed only in kidney from embryonic day 13 to adult and was seen primarily in proximal tubule cells of the outer medulla. Transgene expression and activity were unchanged in response to TGF-β and remained restricted to kidney. Thus we have identified a promoter region within the PAI-1 gene that targets transgene expression to kidney but, unlike the native promoter, is unresponsive to TGF-β in the experimental protocol used.

PLASMINOGEN ACTIVATORS lead to the generation of plasmin from the zymogen plasminogen. Plasmin degrades plasma fibrin and extracellular matrix components either directly or indirectly through the activation of matrix metalloproteases (24). Plasminogen activator inhibitor type 1 (PAI-1) is the physiological inhibitor of both tissue-type and urokinase-type plasminogen activators and plays a pivotal role in the regulation of a variety of fibrinolysis-dependent biological processes, such as embryogenesis, tumor invasion, and angiogenesis (18). PAI-1 suppresses fibrinolytic activity and therefore may be important in tissue remodeling after injury (18). Plasmin PAI-1 is elevated in conditions associated with thrombosis, such as unstable angina and hemolytic uremic syndrome (3). Conversely, reduced PAI-1 activity is observed in conditions characterized by uncontrolled bleeding (18).

PAI-1 mRNA expression is regulated by a variety of conditions in vivo, including endothelial cells, vascular smooth muscle cells, kidney mesangial and tubular epithelial cells, fibroblasts, and hepatocytes (18). In vivo, PAI-1 mRNA expression is observed in many tissues, including heart, lung, liver, and adrenal, and is induced in these tissues by cytokines, such as transforming growth factor-β (TGF-β; see Ref. 18). Under basal conditions in vivo, PAI-1 mRNA is expressed in endothelial cells and smooth muscle cells lining blood vessels but not in tissue parenchymal cells (18). In kidney, PAI-1 mRNA has been localized primarily to endotheial cells (18). In models of renal disease characterized by increased synthesis of extracellular matrix, such as proliferative glomerulonephritis, lupus nephritis, and endotoxemia, PAI-1 mRNA is expressed in parenchymal cells within the affected areas, i.e., mesangial cells, parietal epithelial cells, and tubular epithelial cells, as well as in endothelial cells (1, 15, 22).

To date, information about the PAI-1 gene promoter has been derived exclusively from results of transient transfections in cultured cells. A complex array of regulatory elements, including TGF-β-responsive sequences, confer regulated expression to linked reporter genes, have been identified in the 5′-upstream region of the PAI-1 gene within 1 kb of the transcriptional start site (6, 30). To delineate regulatory elements that target expression in vivo, we generated transgenic mice that carry a hybrid gene comprising the 5′-upstream sequences of the PAI-1 promoter, previously characterized in transfection studies (30), linked to a bacterial β-galactosidase (β-Gal) coding block. We show that these 5′-upstream sequences target transgene expression in vivo exclusively to kidney, primarily to proximal tubules. However, the transgene does not confer TGF-β responsiveness in kidney in the experimental conditions used.

MATERIALS AND METHODS

Construction of a PAI-1-LacZ fusion gene. A hybrid gene was constructed that contained the PAI-1 promoter linked to β-Gal coding sequences in the plasmid pBluescript SK. A BstYI/EcoRI fragment of the human PAI-1 gene encompassing sequences between −1,272 and +75 (30) was ligated using XbaI linkers to a HindIII/BamHI fragment containing the Escherichia coli gpt-trpS-LacZ fusion gene and the Simian virus 40 polyadenylation signals derived from pCH110 (13). The structure of the transgene is shown in Fig. 1. The prokaryotic translation initiation signals, supplied by the short E. coli gpt fragment at the 5′-end of the LacZ fusion gene are functional in eukaryotic cells (8).

The DNA sequence of the 5′- and 3′-ends of the transgene and the PAI-1-LacZ junction was confirmed by standard techniques (29). Enzymatically active β-Gal was expressed in Hep G2 cells transfected with the PAI-1-LacZ plasmid as determined by the o-nitrophenyl-β-D-galactoside conversion assay (25), indicating that the transgene was functional (data not shown). The transgene was released from vector sequences as a 5,102-bp BamHI fragment, recovered on glass-milk resin (Bio-101) after agarose gel electrophoresis, and diluted to a final concentration of 2 µg/ml in injection...
buffer [0.15 mM EDTA and 10 mM tris(hydroxymethyl)amino-
methane (Tris)-HCl, pH 7.4] before injection into oocytes.

Generation and identification of transgenic mice. The trans-
gene was microinjected into male pronuclei of one-cell fertil-
ized mouse embryos obtained from superovulated FVB/N
female mice (Harlan Sprague Dawley, Indianapolis, IN). The
embryos were subsequently transferred to pseudopregnant
Swiss Webster females with the use of standard techniques
(14).

To identify transgenic founders and distinguish transgene
integration sites and orientation, high-molecular-weight ge-
nomic DNA was extracted from a 1.0-cm portion of tail by
digestion at 55°C for 12 h with proteinase K at a final
concentration of 0.5 mg/ml in digest buffer [0.05 M Tris·HCl,
pH 8.0, 0.1 M NaCl, 0.1 M EDTA, 1.0% sodium dodecyl sulfate
(SDS)]. The tail digests were extracted with phenol and
chloroform, and the DNA was precipitated with ethanol.
Southern blot hybridization analysis was then performed
with genomic DNA digested with EcoRI, fractionated by
agarose gel electrophoresis, and transferred to nylon
membranes. Prehybridization was performed at 65°C for at least 1
h in a solution of 1.0 M NaCl, 1% SDS, and 10% dextran.
Hybridization was performed for 12 h at 65°C in the same
solution with 300 µg/ml salmon sperm DNA and [32P]dCTP
probe (the 5,102-bp BamH I transgene fragment). The mem-
branes were washed sequentially in 2× saline sodium citrate
(SSC) at room temperature, 2× SSC with 1% SDS at 65°C,
and 0.1× SSC with 1% SDS at 65°C and were exposed to
X-ray film. Transgenic mice were maintained in the hemizy-
gous state by mating founders with FVB/N mice. The mice
were housed in a pathogen-free barrier facility.

Processing and histological staining for LacZ. Organs were
surgically removed from anesthetized postpartum day 21
(P21) mice (28), fixed in 4% paraformaldehyde in phosphate-
buffered saline, incubated in sucrose, and embedded in Tissue
Tec (Miles) optimum cutting temperature compound for fro-
zen sectioning. Sections (12 μm) were placed on positively
charged slides and stained with nuclear fast red (25). Sections
were then dehydrated in ethanol and coverslipped.

The whole-mount metanephroi were incubated overnight at
0.1% glutaraldehyde in phosphate-buffered saline for 15 min.
Metanephric kidneys were surgically removed and fixed in
4% paraformaldehyde in ice-cold 0.1 M sodium cacodylate,
and the kidneys were embedded in optimal cutting tempera-
ture (OCT) compound. Sections of 7 µm were placed on
positively charged slides and stained with nuclear fast red
(25). Some of the metanephroi were fixed in Bouin’s fixative
at 4°C and stored in 70% ethanol at 4°C until paraffin embed-
ded. Sections of 7 µm were placed on positively charged
slides, fixed for 20 min in 1.25% glutaraldehyde in
phosphate-buffered saline, incubated for 6 h in a solution of
0.5 mg/ml X-Gal, 44 mM N-2-hydroxyethylpiperazine-N’-2-
ethanesulfonic acid, 3 mM potassium ferrocyanide, 3 mM
potassium ferricyanide, 15 mM NaCl, and 1.3 mM MgCl2 at
37°C, and stained with nuclear fast red or eosin, as indicated.

Analysis of tissue β-Gal activity. Transgenic progeny
of founder mice were analyzed for expression of β-Gal reporter
activity in tissues (20). For each line, at least two littermates
were examined. In some experiments, animals were treated
with recombinant TGF-β (50 µg/kg body wt; Collaborative
Biomedical, Bedford, MA) via tail vein injection. β-Gal activ-
ity was quantified in tissue homogenates using the o-
nitrophenyl-d-galactoside conversion assay (25). All measure-
ments of enzyme activity were made within the linear range
of the assay, determined with the use of purified enzyme
(β-Gal from E. coli; Sigma Chemical, St. Louis, MO). Units for
absorbance at 410 nm were converted to nmol o-nitrophenol
by using o-nitrophenol (Sigma) as a standard. Results are
expressed per milligram protein in homogenates.

Northern blot analysis. To determine the expression of
endothelial PAI-1 mRNA, tissues were excised, snap-frozen
in liquid nitrogen, and stored at −70°C. Total cellular RNA
was isolated with the Ultraspec RNA isolation system (Bio-
tec, Houston, TX). RNA (20 µg) was separated on 1.2% agarose-2.2 M formaldehyde gels and transferred to Zeta
Probe membranes (Bio-Rad, Hercules, CA). A 3.2-kb Sac I/Hind III fragment of the mouse PAI-1 cDNA (23) and a
548-bp Xba/Hind III fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (American Type Culture Collection no. 57090) were used as templates to generate specific probes labeled with $[^{32}P]dCTP$ (Amersham, Arlington Heights, IL) by random priming according to the manufacturer's instructions (Prime-It II; Stratagene, La Jolla, CA). Hybridization for both PAI-1 and GAPDH were carried out simultaneously using $3.0 \times 10^6$ and $1.0 \times 10^6$ counts·min$^{-1}·$ml$^{-1}$ of hybridization buffer, respectively. The hybridization and washing conditions used were those recommended by the manufacturer.

Ribonuclease protection assay for β-Gal mRNA. A riboprobe template was generated by polymerase chain reaction amplification of the transgene using oligonucleotide primers designed to include RNA polymerase promoter sequences at the 5'-end. The primers used were 5'-GAATTCTAA TACGACTCACTATAGGGTGCGCAACCTGAATG-3' for sense and 5'-GGATCCATTTAGGTGACACTATAGAACAACGCGACGACCCACGATGATGATCTACGATC-3' for antisense. The underlined sequences refer to the promoters for T7 and SP6 RNA polymerase, respectively. After 35 cycles of polymerase chain reaction (94°C for 30 s, 50°C for 1 min, and 72°C for 2 min), the template was purified after electrophoresis on a 1.2% agarose gel (Quiaquick; Qiagen, Chatsworth, CA) to yield a 472-bp fragment. A BLAST search of the amplified sequence showed no nucleotide identity with any mammalian gene sequence, including mammalian β-Gal. Antisense riboprobe was generated using SP6 RNA polymerase (Stratagene) and 50 µCi $[^{32}P]dCTP$ (800 Ci/mmol; New England Nuclear, Boston, MA). β-Gal mRNA was assayed in kidney by solution hybridization/ribonuclease protection as previously described (2). Total cellular RNA from kidney (20 µg) was ethanol precipitated and dissolved in 15 µl hybridization buffer (80% formamide and 100 mM Tris, pH 7.4, with 300 mM NaCl) containing $0.6 \times 10^6$ counts/min of antisense riboprobe for 15 min at room temperature, heated to 90°C for 10 min before hybridization at 45°C for 16 h. Unprotected RNA was digested with ribonuclease T1 (100 units; Boehringer Mannheim, Indianapolis, IN) for 30 min. The reaction mixture was subsequently incubated with proteinase K and ethanol precipitated. A protected fragment was identified by analyzing the resulting precipitate on a sequencing gel and exposing the gel to Amersham MP autoradiography film.

RESULTS

Generation of PAI-1-LacZ transgenic mice. A 5,102-bp BamHI fragment of DNA (Fig. 1), comprising human PAI-1 upstream sequences fused to the coding sequence of an E. coli gpt-trpS-LacZ fusion gene, was microinjected into fertilized mouse eggs. Southern blot analysis of DNA from tail biopsies revealed that 12 out of 218 live born mice carried the transgene. Analysis of DNA from the progeny of the 12 founders showed that the transgene was transmitted in a Mendelian fashion (50% hemizygotes). The progeny were analyzed for expression of the transgene.

Tissue and cell-specific expression of the PAI-1-LacZ transgene. To determine the tissues and cells that expressed the PAI-1-LacZ fusion gene, adult transgenic progeny of each founder were killed, and their organs were processed for histochemical staining. Sections
were prepared from brain, heart, lung, liver, kidney, intestine, testes, skeletal muscle, and adipose tissue. β-Gal activity was detected by incubating tissues in the presence of the chromogenic substrate X-Gal. Tissues from 2 of the 12 lines of mice showed expression of the transgene. Southern blot analysis of genomic DNA digested with EcoRI, which cuts two times within the transgene, showed that each of the LacZ-expressing lines had different transgene integration sites (Fig. 1). Analyses of tissues from both lines of mice demon-

Fig. 3. Expression of the PAI-1-LacZ transgene in kidney. Whole metanephroi (A–H) or kidneys (I–K) were removed from transgenic mice (B, D, F, H, J, and K) or nontransgenic littermates (A, C, E, G, and I) stained for LacZ (A–K) and counterstained with nuclear fast red (I–K), as described in MATERIALS AND METHODS. A and B: Embryonic day 13 (E13) metanephroi (arrowheads show LacZ-positive cells); C and D: E14 metanephroi; E and F: E15 metanephroi; G and H: E16 metanephroi; I and K: kidneys from P21 mice. H: bar = 0.4 mm for A–H. I: bar = 0.75 mm for I and J. K is an enlargement of a portion of J.
strated that the transgene was expressed exclusively in kidney and not in other tissues (Fig. 2). β-Gal activity was not detected in kidney obtained from nontransgenic littermates (Fig. 3) or in any other organ (not shown).

To provide insight into the ontogeny of transgene expression, we analyzed LacZ expression in kidney obtained from mouse embryos and adult mice in which kidneys were fully developed (P21). In developing kidney, LacZ-expressing cells were first detected on embryonic day 13 (E12.5; Fig. 3B, arrowheads). Expression increased from E14 to E16. At P21, expression occurred primarily in the outer stripe of the outer medulla (Fig. 3) in what appeared to be the S3 portion of the proximal tubule (Fig. 3K). LacZ-expressing cells were not observed in the glomerulus or in vascular endothelial or smooth muscle cells. However, scattered LacZ-expressing cells were detected elsewhere in the renal cortex and inner medulla (Fig. 3K). The identity of these cells has not been determined. No staining for LacZ was detected in kidneys from nontransgenic littermates (Fig. 3, A, C, E, G, and I).

Because the expression of PAI-1 is known to be low in many cells and tissues and the mRNA is detectable only after induction by cytokines such as TGF-β, we sought to determine whether treatment of mice with TGF-β would increase the expression of the PAI-1-LacZ transgene in kidney or alter the pattern of expression of the transgene in cells and tissues. To determine the optimal dose of TGF-β, nontransgenic mice were administered either TGF-β (25–50 µg/kg) or vehicle via tail vein injection. Tissues were harvested after 3 h when TGF-β-induced expression of endogenous PAI-1 mRNA was shown to occur (18). The 25 µg/kg dose of TGF-β, although lower than that used by others (7), was sufficient to induce a peak response of endogenous PAI-1 mRNA in heart, lung, spleen, and adipose tissue, whereas higher doses actually resulted in less induction of the mRNA in these tissues (Fig. 4). Only kidney and skeletal muscle PAI-1 mRNA increased in response to higher doses of TGF-β. We chose a final dose of 50 µg/kg to maximize our ability to detect induction of PAI-1 mRNA in as many tissues as possible.

To correlate the response of endogenous PAI-1 mRNA with that of the transgene, transgenic animals were killed 3 h after injection of TGF-β (50 µg/kg), an interval sufficient to see expression of linked β-Gal transgenes (16) and endogenous PAI-1 mRNA (Fig. 4), and their tissues were excised for analysis of PAI-1 mRNA and β-Gal activity. PAI-1 mRNA was not detectable in kidney from PAI-1-LacZ mice treated with vehicle when analyzed by Northern blot (Fig. 5). However, the 3.2-kb PAI-1 mRNA was detectable in kidney from all five mice that received TGF-β1 (Fig. 5). Tissues from these same animals were also prepared for histochemical analysis of transgene expression. Despite induction of endogenous PAI-1 mRNA in response to TGF-β1 administration, PAI-1-LacZ transgene activity was still undetectable in all tissues except kidney. Within the kidney, the pattern of expression of the transgene did not change (data not shown). Because the detection of transgene expression using X-Gal is not quantitative, we measured the expression of β-Gal in kidney tissue homogenates using the colorimetric o-nitrophenyl-D-galactoside conversion assay. The level of β-Gal activity was not different in kidneys from TGF-β-treated mice when compared with vehicle-treated controls (Student’s t-test; Fig. 6). Conversion of the substrate was not detected in renal homogenates from nontransgenic littermates (data not shown). The finding that TGF-β failed to induce transgene expression was further verified by performing ribonuclease protection assays for bacterial β-Gal. A 420-bp fragment of β-Gal mRNA was protected by RNA from kidneys of transgenic mice (Fig. 7). Expression was unaltered in kidney of transgenic animals treated with TGF-β compared with sham-injected controls (Fig. 7).

Fig. 4. Northern blot analysis of PAI-1 mRNA. Nontransgenic mice received injections of either saline vehicle (0) or 25 (1) or 50 (2) µg/kg transforming growth factor (TGF)-β1 peptide by tail vein 3 h before death. Total RNA was extracted from organs, and Northern blot analysis was performed as described in MATERIALS AND METHODS. Simultaneous hybridization was performed for detection of PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
DISCUSSION

The kidney is an important site of PAI-1 expression. Although basal levels of renal PAI-1 mRNA are low, levels increase in response to treatment of mice with inflammatory cytokines such as TGF-β (18). This is of potential relevance to renal pathophysiology, because, in several disease settings, PAI-1 has been implicated as a mediator of fibrin deposition.

The development of glomerulosclerosis is characterized by a marked decrease in glomerular plasminogen activator activity and a dramatic increase in synthesis and deposition of PAI-1 in glomerular matrix (1). In MRL/lpr mice, a well-characterized animal model of lupus nephritis, PAI-1 mRNA is expressed at high levels at sites of crescent formation in glomeruli, in epithelial cells within areas of active interstitial nephritis, and in endothelial cells at sites of necrotizing arteritis (15). The development of hemolytic uremic syndrome, accompanied by deposition of fibrin in glomeruli (3), is characterized by elevated levels of PAI-1 in plasma. Removal of PAI-1 from the circulation by dialysis improves renal function (3).

PAI-1 expression is regulated by TGF-β. Each of the models of renal disease described above is associated with increased levels of renal TGF-β (2). Where examined, enhanced expression of TGF-β precedes the elevation of PAI-1 mRNA (21, 22). A causative pathophysiological role for TGF-β-enhanced PAI-1 is supported by the finding that administration of anti-TGF-β antibodies to glomerulonephritic rats inhibits both the deposition of PAI-1 into the mesangial cell matrix and the associated glomerulosclerosis (2).

Presently, we have shown targeted expression of a LacZ reporter exclusively to the kidney, primarily in the S3 segment of the proximal tubule in transgenic mice carrying 1.3 kb of the 5’-upstream promoter sequence of PAI-1. The same pattern of transgene expression limited to kidney tubule cells and seen in two independent lines of mice from different founders with different transgene insertion sites indicates that tissue-specific expression was not dependent on the flanking sequences adjacent to the insertion site of the transgene but rather due to sequences within the transgene. Although the kidney is an important site of PAI-1 production in inflammatory states (18), this observation was unexpected. The sequence used to construct our transgene drives expression of reporter genes in many different cells in culture, including several types of endothelial cells, hepatocytes, and renal tubular epithelial cells (6, 18, 30). It would appear that our transgene lacks upstream silencer elements that normally suppress expression in proximal tubule cells in the absence of inflammatory stimuli.

PAI-1-LacZ transgene expression in kidney was not detected in endothelial cells or smooth muscle cells of blood vessels, which express low levels of PAI-1 under basal conditions (18), or within glomerular epithelial and mesangial cells, which express PAI-1 in inflammatory states (1, 15, 18, 21, 22). Because TGF-β mRNA and peptide are present in S3 proximal tubules (2) and may facilitate transgene expression at this site, we asked whether exogenous TGF-β administration would enhance the expression of the transgene in other tissues and cells. Although we and others have shown that endogenous PAI-1 mRNA expression is enhanced by the administration of TGF-β1 to mice, we did not detect any alterations in transgene expression after stimulation with TGF-β. These results should be interpreted with some caution because it is possible that transgene expression may be responsive to higher...
doses of TGF-β or that transgene expression might be enhanced at a different time after injection of TGF-β.

Data from transient transfection studies indicate that the DNA sequences within the transgene we used can confer TGF-β sensitivity to linked reporter genes (6,18,30). However, based on those studies and the findings presented here, it appears that the DNA sequences necessary for TGF-β-induced PAI-1 responsiveness in vivo and for expression in cells other than the proximal tubule lie outside the region contained within our transgene. The discrepancies between the in vitro and in vivo systems may lie in the fact that PAI-1 gene expression is altered in tissue culture. PAI-1 expression is relatively low in endothelial cells in vivo and is markedly enhanced within hours after the cells are placed in culture (18). Alternatively, the results may be due to the fact that, in transgenic models, the promoter/reporter construct is stably integrated into the genome. All prior analyses of the PAI-1 promoter were based on transient transfection studies. To our knowledge, there have been no studies of the PAI-1 promoter using stable transfections in cultured cells, which demonstrate altered promoter/reporter activity compared with transient transfections (9). With this in mind, it is important to note the recently published observations of Dong et al. (7), in which a similar PAI-1 promoter/reporter construct was introduced into rat carotid arteries with the aid of a “nonintegrating” adenovirus vector. In their study, the expression of the transgene was observed within the carotid endothelium and was induced by TGF-β treatment (7). It is possible that the transfection of endothelial cells in vivo with a nonintegrating adenovirus vector is similar to transient transfections performed in cultured cells and allows for expression of the transgene that may not reflect gene expression in vivo. Taken together with the observations presented here, it is clear that more studies are required to decipher the additional regulatory sequences that target PAI-1 gene expression to cell types other than renal proximal tubule cells and mediate PAI-1 gene induction in vivo in response to TGF-β.

There are other examples of promoters that target expression of linked transgenes to proximal tubule cells. These include the phosphoenolpyruvate carboxykinase (17), γ-glutamyltranspeptidase type I (26), and erythropoietin (19) gene promoters. Like PAI-1, the endogenous products of these genes are expressed in multiple cell types in different tissues. Analyses of transgenic animals have shown that cell-specific elements overlap, as transgene expression was also directed to other cells and tissues. To the best of our knowledge, most promoters with potentially high specificity for kidney that have been used to generate transgenic animals also drive expression of the transgene to other sites (4, 11). Exceptions include the renin gene (10), a transgene constructed from a truncated β-lactoglobulin promoter (5), and a transgene constructed from a 346-bp fragment of the mouse γ-glutamyltranspeptidase type II promoter (27). The expression of the renin transgene was restricted to epithelial juxtaglomerular cells. Although expression of the β-lactoglobulin transgene was restricted to kidney, the specific types of cells that express the transgene have not yet been determined. The γ-glutamyltranspeptidase transgene and the PAI-1 transgene are, to the best of our knowledge, the only examples of transgenes targeted primarily to the proximal tubule. The PAI-1 transgene is the first example of a transgene targeted primarily to a specific segment of the proximal tubule. Such selective targeting and the fact that transgene expression occurs during kidney development offer the potential to use transgenic animals to study the ontogeny of S3 proximal tubule cells to study the effect of gene expression within a specific population of renal tubule epithelial cells and to aid in the construction of cell-specific expression vectors for renal gene therapy.

We thank Marilyn Leung, Nancy Brada, Hainy Atallah [American Heart Association (AHA), Missouri Affiliate Summer Fellow], and Michael Fleischli for technical assistance. We are grateful to Drs. Jeffrey Saffitz and John M. Kissane at Washington University for reviewing the histology slides and to Kelly Hall for secretarial assistance.

M. P. Emert and J. G. Rogers were supported by the AHA, Missouri Affiliate. D. P. Basile, C. M. Sorenson, and M. R. Hammerman were supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant DK-02716. M. R. Hammerman was also supported by NIDDK Grant DK-45181. C. M. Sorenson was also supported by a Scientist Development grant from the AHA. J. J. Billadello is an Established Investigator of the AHA.

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Received 14 March 1997; accepted in final form 5 November 1997.

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