Pituitary-Specific Transcription Factor (Pit-1) Binding Site in the Human Renin Gene 5′-Flanking DNA Stimulates Promoter Activity in Placental Cell Primary Cultures and Pituitary Lactosomatotropin Cell Lines

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Abstract Renin gene expression is limited to a number of specific tissues, including the kidney, adrenal glands, reproductive organs (of particular relevance to this study, the placenta), and the pituitary gland. In the present study, we investigated the human renin (hRen) 5′-flanking DNA sequences required to drive the expression of a luciferase reporter gene in placental and pituitary cells and in two cell lines, 293 and JEG-3, which have been proposed as model systems with which to study transcriptional regulation of renin genes. The activities of specific sequences in the hRen 5′-flanking DNA sequences in human placental cell primary cultures were very similar to those that we previously reported in pituitary cells, suggesting the involvement of common promoter elements and related transcription factors. Accordingly, the binding site for the pituitary-specific transcription factor (Pit-1) was the major determinant of renin promoter activity in both pituitary and placental cells. Gel mobility shift analysis showed a placental nuclear factor with a gel mobility different from that of Pit-1. However, Northern blot analysis failed to demonstrate abundant Pit-1-related mRNAs in renin-expressing cultures of chionic and decidual cells, suggesting that the placental factor is not closely related to Pit-1. Although a factor from 293 cells also bound to the Pit-1 site, it had gel mobility shift characteristics different from Pit-1 and the placental factor. Moreover, the low promoter activity in 293 cells was independent of this site or, indeed, of sequences upstream from the TATA box. In JEG-3 cells, renin 5′-flanking DNA sequences showed virtually no transcriptional activity. Taken together, these data suggest that common cis-acting sequences direct renin gene expression to pituitary and placental cells and that the activity of these sequences is dependent on the presence of transcription factors specific to these cells. Our studies also indicate that 293 and JEG-3 cell lines may not be appropriate for the study of renin gene expression. (Circ Res. 1994;75:624-629.)

Key Words • renin gene expression • pituitary • placenta • transcription factor

As the rate-limiting enzyme in the angiotensin-aldosterone cascade, renin, released from renal juxtaglomerular (JG) cells, plays a central role in determining blood pressure and electrolyte homeostasis.1 Although elevated blood pressure should suppress renin release from JG cells, a spectrum of renin levels is found in hypertensive patients. Those with high renin levels have been shown to have a higher incidence of myocardial infarction and stroke.2,3 Because renin release must be accompanied by de novo synthesis to replenish depleted JG storage granules, the mechanisms that direct renin gene expression are likely to play an important role in the pathogenesis and sequelae of hypertension. Understanding these mechanisms may provide better approaches to the treatment of hypertension.

Studies in transgenic mice have broadly defined the renin genomic sequences required for JG cell–specific expression.4-7 However, in the absence of JG cell lines, it has been difficult to identify the specific cis-acting sequences involved and the trans-acting factors with which they interact. Since renin is also expressed in the pituitary gland8,9 and the placenta,10 cell culture systems from these tissues have been used to study the transcriptional mechanisms that direct renin gene expression.11-14 Using renin-luciferase hybrid genes transfected into the pituitary tumor cell line GC, Sun et al14 demonstrated that human renin gene (hRen) 5′-flanking DNA sequences contain both positive and negative regulatory elements. Sequences contained between −148 and +18 (numbered relative to the transcription start site at +1) were found to be sufficient for maximal expression. Within this region, binding of the pituitary-specific transcription factor (Pit-1) was shown to be the major determinant of promoter activity.14 In studies using primary cultures of placental decidual cells, sequences contained between −100 and +18 were also found to be sufficient for the expression of renin-CAT hybrid genes.12 However, the elements involved were not further delineated.

In other studies, transfection of JEG-3 choriocarcinoma cells with CAT constructs containing hRen 5′-flanking sequences linked to the herpes simplex virus thymidine kinase (HSV-TK) promoter indicated the...
presence of both positive and negative regulatory elements.\textsuperscript{11,13} More recently, mouse \textit{Ren-I}\textsuperscript{F} 5'-flanking DNA sequences were shown to be transcriptionally active in the human embryonal kidney cell line 293.\textsuperscript{15} Although a 293 cell nuclear factor bound to the conserved Pit-1 site, activation by this element was weak unless a sequence contained within a 476-bp insertion specific to mouse renin genes was also present.

Although these studies identified a number of positive and negative regulatory elements, the level of resolution at which regulatory regions in the renin 5'-flanking DNA were mapped was not sufficient to determine whether common mechanisms direct renin gene expression to these different tissues. The identification of common mechanisms would simplify studies of the molecular mechanisms and provide evidence for common pathways through which extracellular signals might regulate renin gene expression.

In the present study, we compared the promoter activity of \textit{hRen} 5'-flanking DNA sequences in human placental cell primary cultures and in JEG-3 and 293 cell lines. We also compared binding of transcription factors from each of these cell preparations to the Pit-1 site. These studies show that although common cis-acting sequences appear to direct transcription in both placental and pituitary cells, activity in these two cell types appears to be mediated by different trans-acting factors. In contrast, renin promoter activity in JEG-3 and 293 cells appears to be due to basal transcription from the TATA box, independent of 5'-flanking DNA sequences further upstream.

Materials and Methods

Cell Cultures of Chorionic Laeve and Decidua

Chorionic or decidual cells were prepared by using modifications of established protocols.\textsuperscript{16,17} Term placentas were obtained from consenting patients by following a protocol approved by the New York Hospital–Cornell Medical Center Human Ethical Review Committee. Within 30 minutes after delivery, placental membranes were rinsed free of blood with 0.9% saline at room temperature. After removing the amniotic layer, the decidual layer was gently scraped off with a straight-edged razor blade. Chorionic and decidual tissues (10 to 20 g each) were incubated separately in 50 to 100 mL of Hank's balanced salt solution (calcium and magnesium free, CMF-H) containing 0.05% trypsin (Sigma type I) and 0.05% collagenase (Worthington type CLS II). After a 20-minute digestion, 10 mg DNase I (Sigma type IV) was added, and the incubation continued for 10 minutes. Tissue pieces were allowed to settle, the medium was decanted and mixed with an equal volume of calf serum, and the cells were pelleted for 10 minutes at 50g. This procedure was repeated twice. Medium from the first digestion contained mainly erythrocytes and cell debris, which were discarded. Cells from the second two incubations were combined in 5 mL CMF-H and applied to a Percoll step gradient (15% and 40% steps in CMF-H) and centrifuged at 1200g for 20 minutes. Cells banding at the interface were collected, washed three times in CMF-H, and plated at a density of \textasciitilde10\textsuperscript{5} cells/mL in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). JEG-3 and 293 cells were obtained from the American Type Culture Collection and grown in HEPES-DMEM containing 10% FBS. The use of these cells in studies of renin promoter activities has been described by other researchers.\textsuperscript{11,15,18}

Immunocytochemistry and Renin Assay

An antiserum to purified recombinant human renin was prepared in rabbits. Serum obtained after the fourth booster was used in these studies. This antiserum stained intensely the juxtaglomerular apparatus (JGA) of normal mice and of human kidneys with advanced arterial nephrosclerosis. It also stained a renal JGA tumor. Preincubation of the antiserum with highly purified recombinant human prorenin abolished staining to sections of placental tissue and to chorionic cell monolayers (authors' unpublished data). An anti-\textalpha-actin monoclonal antibody was obtained from Boehringer Mannheim.

Cell cultures at or near confluence were fixed briefly in Bouin's solution (Polyscientific) and then dehydrated through a series of graded ethanol incubations. Anti-renin and anti-\textalpha-actin antisera were applied at dilutions of 1:1000 and 1:20, respectively. After incubation for 16 hours at 4°C, the monolayers were washed in phosphate-buffered saline. Bound antibody was localized by using an Immunogold kit (Amersham) with a 1-nm gold particle size. Renin activity in cell culture medium was assayed as previously described.\textsuperscript{19}

Constructions and Transfections

Renin-luciferase hybrid genes used in the present study are described in detail elsewhere.\textsuperscript{14} These constructions contain \textit{hRen} sequences extending from +18 to various positions in the 5'-flanking DNA up to \textasciitilde2748. Two of the constructions contain mutations in the Pit-1 site that reduce or prevent Pit-1 binding (\textasciitilde14841 and \textasciitilde9842).\textsuperscript{14} Transfections of placental cells and 293 and JEG-3 cell lines were carried out by the calcium phosphate method\textsuperscript{20} on 60-mm dishes of each cell preparation at \textasciitilde50% confluence. Eight micrograms of DNA was applied to each dish for 12 to 16 hours, after which the medium was replaced. Cells were harvested 48 hours after transfection and lysed in 200 \muL luciferase assay buffer, of which a 100-\muL aliquot was assayed for activity, pSV\textasciitilde4/luc,\textsuperscript{21} which contains the Rous sarcoma virus long terminal repeat, was transfected in parallel, and the data were normalized to the activity of this construct.

Gel mobility shift assays were carried out with nuclear extracts prepared from cell cultures as described previously\textsuperscript{14} by using a \textit{32P} end-labeled oligonucleotide corresponding to positions \textasciitilde80/\textasciitilde58 in the \textit{hRen} 5'-flanking DNA.

Northern Blot Analysis

RNA was prepared by using chaotropic agents.\textsuperscript{22} Total RNA (10 \mug per lane) was fractionated on a 1.2% agarose gel containing 20 mmol/L MOPS (pH 7.0) and 2.4% formaldehyde. Size markers run in parallel were from GIBCO-BRL. After electrophoresis, RNA was transferred by blotting in 10x standard saline citrate (SSC) to a nylon membrane, which was then dried at 60°C for 1 hour and UV-crosslinked for 30 minutes. Membranes were prehybridized and hybridized in solutions containing 5x SSPE, 50% formamide, 0.2% sodium dodecyl sulfate (SDS), 100 \mug/mL denatured salmon sperm DNA, and 5x Denhardt's solution. Probe was labeled by random priming of a 369-bp fragment spanning the DNA binding domain of Pit-1 (amino acids 141 through 263).\textsuperscript{23} Hybridization was at 37°C for 24 hours, and the membrane was then washed two times for 20 minutes in 1x SSC and 1% SDS at 20°C, 47°C below the calculated melting temperature (\textit{Tm}).

Results

Renin Secretion and Synthesis by Primary Cultures of Chorionic and Decidual Cells

Cell preparations from both chorion laeve and decidua grew rapidly and achieved confluence after 5 to 7 days in culture. At confluence, both chorionic and decidual cell cultures secreted prorenin activity of 10 000 to 20 000 ng angiotensin I \textcdot mL\textsuperscript{-1} h\textsuperscript{-1} in 24 hours. These cultures remained viable for up to 7 weeks and continued to secrete prorenin through two to three passages.
Decidual cell cultures contained mostly elongated cells, whereas chorion laeve cultures exhibited a number of different cell morphologies, including irregular polygonal and elongated cells (Fig 1). In chorion laeve cultures, overall in excess of 70% of cells stained with the anti-renin antibody (Fig 1A). Of these, a smaller population (5% to 15%) stained intensely. The broad, flattened morphology of these strongly staining cells suggests that they might be more mature cells, which express high levels of prorenin. Most of the cells contained in these preparations also bound to antibodies specific for the smooth muscle-specific marker α-actin (Fig 1B). Actin staining in larger cells often exhibited filamentous structures typical of the organization of actin into contractile bundles (Fig 1B), suggesting that renin-producing cells in these cultures are derived from smooth muscle cells.

### hRen Promoter Activity in Placental Cell Primary Cultures and JEG-3 and 293 Cell Lines

To compare the activity of hRen 5′-flanking DNA sequences in different cell types, renin-luciferase hybrid genes were transfected into primary cultures of chorion laeve and decidua and JEG-3 and 293 cell lines (Fig 2).

In secondary cultures of chorion laeve, the most active hRen construct contained sequences −148/+18. This construct exhibited 1% of the activity of pRSV.luc, which yielded 3×10⁵ to 7×10⁵ light units in the luciferase assay in the experiments shown. Generally, we found that cotransfection of an expression vector to normalize transfection efficiencies suppressed the activity of the hRen promoter. Therefore, activities were normalized to pRSV.luc transfected in parallel dishes. Data are shown for preparation 9 (HC9). However, similar results were obtained in four separate experiments by using cells from different placentas and at least two different preparations of a number of key constructs. The mean±SEM values for these replicate experiments are shown superimposed on the results obtained with HC9.

The relative activities of the various hRen 5′-flanking DNA sequences were similar in chorion laeve and GC cells (Fig 2), suggesting that common promoter elements are involved. Deletion of sequences between −2748 and −148 led to increased activity, whereas mutagenesis or deletion of the Pit-1 binding site markedly reduced activity. As in GC cells, loss of the Pit-1 site caused the greatest reduction in activity of any...
mutation. Although deletion of sequence between −148 and −98 also resulted in losses in activity, mutagenesis of the Pit-1 site resulted in the same loss of activity as deletion of the entire region −148 to −65, suggesting that any positive effect of sequences contained between −148 and −98 is dependent on the Pit-1 site. This is almost identical to the pattern of activities observed in GC cells (Fig 2).

hRen luciferase constructs were also tested for promoter activity in 293 and JEG-3 cells. Whereas in chorionic cell primary cultures hRen −148/+18 5′-flanking DNA stimulated luciferase expression 70-fold over that of the promoterless construct pZLuc, in 293 cells this stimulation was only 17-fold, and in JEG-3 cells it was 1.4-fold. Indeed, in JEG-3 cells, none of the hRen 5′-flanking sequences stimulated luciferase expression more than 2-fold. The modest promoter activity of hRen 5′-flanking sequences observed in 293 cells was near maximal with the −34 sequence. Since this segment of 5′-flanking DNA contains little more than the TATA box, the activity observed in these cells is most likely due mainly to basal transcription by RNA polymerase independent of the effect of factors bound to sequences further upstream. Similar patterns of basal expression were also found in other choriocarcinoma cell lines, BeWo and JAR (not shown).

Binding of Placental Nuclear Factor(s) to the hRen Pit-1 Site

Gel mobility shift assays were carried out by using the hRen Pit-1 binding site (−80/−58) as the probe, with nuclear extracts from chorion laeve and decidual cells at various passage numbers, and GC and 293 cells (Fig 3). Nuclear extracts from both chorion laeve and decidual cells resulted in a single band (lanes 4 through 10), which ran between the monomer and dimer forms of the Pit-1 complex formed with GC cell nuclear extract (lane 2). Increasing amounts of chorionic cell nuclear extract led to proportional increases in the intensity of this complex (lanes 8 through 10), suggesting high-affinity binding. The specificity of interactions involved in formation of this complex was further demonstrated by the ability of an oligonucleotide containing a strong Pit-1 binding site from the human growth hormone gene to compete for complex formation (not shown). Nuclear extracts from later passages, where the rate of prorenin secretion into the media was reduced (suggesting lower levels of renin gene expression) exhibited proportionally lower formation of this complex (compare lanes 4 and 5). Nuclear extract from 293 cells formed a complex (lane 3) with different mobility to GC, chorionic, and decidual cell extracts, whereas no binding was evident with extract from JEG-3 cells (not shown).

Northern Blot Analysis of POU Gene Expression in Renin-Expressing Tissues

Further studies were carried out to determine whether Pit-1–related sequences are expressed in placental cell cultures. RNA was prepared from chorion laeve and decidual cell primary cultures and subjected to Northern blot analysis with a Pit-1 probe. RNA from GC cells that express the endogenous Pit-1 gene was included as a positive control. Since sequences from the DNA binding domain of the POU family are highly conserved, this region of the Pit-1 cDNA was used as the probe. Northern blot analysis was carried out at low-stringency conditions under which sequences with as little as 50% identity to the POU DNA binding domain would be expected to bind the probe. Although RNA from GC cells (which express high levels of Pit-1) showed a strong band of the expected size (2.4 kb) and a weaker band corresponding to the primary transcript (4.5 kb), RNA from both chorion laeve and deciduaI cell cultures showed only very weak hybridization with the probe (not shown). Although this might indicate that a POU family factor is expressed at low levels, the intensity of the band observed in gel mobility shift assays with chorion laeve nuclear extracts (described above) suggested that the factor involved is highly abundant. Therefore, it seems more likely that the placental cell factor that binds the hRen Pit-1 site is only distantly related to Pit-1.

Discussion

In the present study, we compared the activity of renin-luciferase hybrid genes containing various hRen 5′-flanking DNA sequences in placental cell cultures and a number of cell lines. Further, we compared the binding of nuclear factors from these different cell types to a sequence we had previously shown to bind the pituitary-specific factor Pit-1, the major transcriptional activator of renin gene expression in pituitary cells. The results of these experiments show that (1) hRen 5′-flanking DNA sequences are transcriptionally active in primary cultures of chorion laeve and decidual cells, (2) common 5′-flanking DNA sequences appear to direct hRen promoter activity in placental and pituitary cells, (3) low-level promoter activity in 293 human embryonal kidney cells appears to be largely independent of sequences contained upstream of the TATA box, (4) hRen 5′-flanking DNA sequences are virtually inactive in their ability to drive transcription in human choriocarcinoma cell lines JEG-3, JAR, and BeWo, and
(5) binding of a nuclear factor from placental cells to the Pit-1 site is correlated with promoter activity. Although the placental Pit-1-like factor appeared to be relatively abundant, Northern analysis of placental cell RNA indicated that at the primary sequence level, this factor is at best only distantly related to Pit-1. Taken together, these observations suggest that common promoter elements direct renin gene expression in pituitary and placental cells but do so through interactions involving cell-specific transcription factors.

Renin has been detected both enzymatically and immunocytochemically in chorion laeve and is also found in cell cultures of chorion laeve. Although the source of placental renin remains the subject of conjecture, placentals in culture are the only untransformed cell culture systems that express the human renin gene. Since, in term placentas, chorion laeve is generally more abundant than attached decidua, we characterized renin expression in chorion laeve cultures more extensively. Using a polyclonal antibody raised against highly purified recombinant human renin, we were able to demonstrate renin immunoreactivity in primary cultures of chorion laeve. A large number of cells in these preparations also stained for the smooth muscle–specific marker α-actin. Since >70% of cells stained for each marker, it seems likely that renin-expressing cells in this preparation were either derived from smooth muscle cells or, at least, express common genes. This would imply that common mechanisms may also be involved in directing renin expression to smooth muscle–derived JG cells. Indeed, our preliminary studies with the mouse renal renin-expressing cell line As 4.127 suggest that the cis-acting elements in hRen 5′-flanking DNA that control renin transcription in placental and pituitary cells also function in these renal cells.

Renin and a number of other proteins, such as prolactin and apolipoprotein B (apo B), are unusual in that they are expressed specifically but in a number of different cell types. In the prolactin gene, a downstream promoter interacts with an upstream enhancer to drive lactotropotropic-specific expression. Early in development, the prolactin promoter region appears to be sufficient to direct expression to lactosomatotropic precursor cells, which synthesize both prolactin and growth hormone. Prolactin expression in the placenta, on the other hand, is directed by a distal promoter that is entirely separate from the pituitary-specific control elements. Apo B expression is also controlled by promoter and enhancer elements. However, in this case, different sequences in the proximal promoter region in combination with cell-specific enhancer and repressor sequences situated further upstream direct expression to liver and colon. The findings of the present study implicate yet another mechanism in which common promoter elements interact with different cell-specific transcription factors.

Gel mobility shift assays demonstrated the high-affinity binding of a factor from placental cell nuclei thatcorrelated closely with expression of both endogenous and transfected renin genes. However, low-stringency Northern blot analysis of RNA from chorionic and decidual cells with a probe corresponding to the highly conserved DNA binding domain of the POU family of transcription factors failed to detect any abundant cross-hybridizing mRNAs. Although we cannot rule out a POU family member, the high abundance of the placental factor in relation to the absence of any high abundance POU mRNA would suggest the involvement of a more distantly related factor. Such a relation occurs with the homeobox proteins, which are homologous to the POU family in the DNA binding domain. Although individual POU and homeobox proteins may share as little as 25% identity in their DNA binding domains, these factors all appear to bind the same, or similar, AT-rich sequences.

Whereas a number of different transcription factors may stimulate renin gene expression, not all factors capable of binding the Pit-1 site are either able or sufficient to activate transcription. Although a nuclear factor from 293 cells was found to bind the Pit-1 site, the weak promoter activity of renin 5′-flanking DNA sequences in these cells was independent of this site. Previously, mouse renin promoter activity in 293 cells had been shown to require both the Pit-1 site and a site contained in an insertion element specific to mouse renin genes. Our findings further show that in the absence of sequences resembling the mouse-specific insertion element, there are no other sequences in the human renin 5′-flanking DNA up to position −2748, which can activate basal transcription from the hRen TATA box more than twofold. Thus, hRen promoter activity in 293 cells appears to be due mainly to modest levels of basal transcription originating from the TATA box independent of the influence of promoter elements further upstream. A similar pattern of activities has been described for the apo B gene. Although different cell-specific sequences upstream from position −85 are required for high-level transcription in apo B-expressing hepatoma and colon carcinoma cells, proximal sequences contained downstream from −85 give rise to the highest levels of activity in Chinese hamster ovary cells, which do not express the endogenous apo B gene.

Burt et al., using the JEG-3 choriocarcinoma cell line, demonstrated a twofold stimulation of CAT activity by hRen sequences −892/+13. Since no stimulation was found in a number of other cell lines derived from renin-expressing tissues, the authors proposed that JEG-3 cells might serve as a useful model system in which to study transcriptional control elements in the human renin gene. To create a system in which basal expression was elevated, Burt et al. subsequently linked hRen 5′-flanking DNA sequences to an HSV-TK promoter. These studies suggested that sequences −892/−149 contain positive regulatory elements whose activity is suppressed by sequences −148/+13. In the present study, we found no evidence for negative regulatory elements in the more proximal region. Indeed, in both placental and pituitary cells this region contained the strongest positive control elements, whereas negative elements were contained further upstream. In agreement with the earlier report of Burt et al., we found that hRen 5′-flanking DNA sequences stimulated expression of the reporter gene in JEG-3 cells one- to twofold. However, this activity was independent of the extent of 5′-flanking DNA tested and 50- to 100-fold lower than the stimulation afforded in placental or pituitary cells. Taken together, these observations demonstrate that the effects of renin 5′-flanking DNA sequences on the HSV-TK promoter do not reflect the net effects of these sequences in cells in which the native hRen promoter is active.

Studies using transgenic animals have shown that as little as 0.9 kb 5′-flanking DNA is sufficient for cell-specific
expression of the human renin gene, but only when coding and intervening sequences are also present.6,35 Although these findings show that 5′-flanking DNA sequences proximal to hRen are sufficient for gene expression, sequences downstream from the transcription start site may contain cell-specific transcriptional enhancer elements or contribute to mRNA stability. The low promoter activities of the hRen 5′-flanking DNA sequences that we describe here are compatible with both models. It will be important to determine whether the proximal sequences we have shown to drive maximal renin transcription in placental and pituitary cells are also necessary and sufficient for renal renin gene expression in transgenic models in vivo and to determine the role of genomic sequences in directing renin gene expression both in transfection experiments and transgenic animals. We are currently investigating the nature of the factor(s) that interacts with the hRen Pit-1 site in placental and renal cells and the role of other sequences that contribute to activity such as the region –148/–99.

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