Human Renin Gene*

A Novel Distal Enhancer Confers Chorionic Expression on the Human Renin Gene*

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Stéphane Germain‡, Fabrice Bonnet‡, Josette Philippe, Sébastien Fuchs¶, Pierre Corvol, and Florence Pinet¶

From the INSERM Unité 36, Collège de France, 75005 Paris, France

Renin catalyzes the rate-limiting step of the renin-angiotensin system, which regulates blood pressure and electrolyte homeostasis. To determine cell-specific human renin gene control elements, the transcriptional activity of promoter regions up to position −8876 was studied in renin-expressing cells. A positive regulatory region conferring ~57-fold higher transcriptional activity to the human renin gene promoter in chorionic cells was identified between nucleotides −5777 and −5552. It had the orientation-independent activity typical of classical enhancers. It also conferred ~59-fold higher transcriptional levels from the heterologous simian virus 40 (SV40) promoter in chorionic cells and ~6-fold higher transcriptional levels in Calu-6 and As4.1 cells, whereas no effect was measured in non-renin-expressing cells. DNase I footprinting showed that this enhancer contains three binding sites for chorionic cell nuclear extracts. Functional analysis suggested that the activity of the enhancer is regulated by differential mechanisms in the three renin-expressing cells involving a complex arrangement of AP-1 motifs binding cell-specific members of the basic leucine zipper family of transcription factors. Thus, our results demonstrate that this enhancer plays a key role in the expression of the human renin gene in the chorion and may also be involved in its regulated expression in other tissues.

Renin, a key enzyme of the renin-angiotensin system regulates blood pressure and electrolyte homeostasis. The juxtaglomerular (JG) cells of the kidney are the principal site of renin synthesis, but renin is also synthesized in a variety of nonrenal tissues. Chorionic tissue is one of the main extrarenal sites at which a local renin synthesis has been demonstrated at the protein (1, 2) and the gene levels (3). Therefore, cultured chorionic cells (4) have been a model of choice for studying the regulation of the human renin gene transcription. Efforts to dissect human renin gene transcriptional regulation have focused mainly on the proximal promoter. Duncan et al. (5) demonstrated by transient transfections in chorionic cells that the first 584 bp of the human renin 5′-flanking region are able to direct chloramphenicol acetyltransferase expression. We then studied two elements, the cyclic AMP responsive element (−229 to −220) and a sequence similar to that of homedomain-containing transcription factor binding site, located from nucleotides −79 to −69, which are responsible for basal renin gene promoter activity in chorionic cells (6, 7). We have also shown that renal and chorionic tissues contain similar nuclear binding proteins recognizing the same regions in the human renin proximal promoter (8).

More distal human renin promoter regions up to nucleotide −2824 have been studied but no cis-acting element functionally important for promoter activity was identified (7). These distal regions were less efficient at directing reporter gene expression, suggesting that there may be a discrete silencer between nucleotides −2824 and −892. Transgenic animal studies have also failed to produce a coherent description of the human renin gene control elements in the coding, 3-kb 5′-flanking region and 1.2-kb 3′-flanking region (9–11), demonstrating the need to look for more distal specific cis-regulating elements. Recently, Yan et al. (12) showed that a 220-bp region located 12 kb upstream from the transcription start site of the human renin gene gave up to 47-fold higher transcription rates when associated with the proximal promoter and used to transfect mouse As4.1 cells.

Differential regulation of the proximal promoter has been reported in human renin-producing chorionic cells (7) and Calu-6 cells (13, 14). Therefore, the differential regulation of transcription may be involved in cell-specific expression of the human renin gene in the chorion. We isolated a genomic clone containing the human renin gene and its surrounding sequences. The functional activity of the promoter until position −8876 was determined by transient transfections of human chorionic cells, Calu-6 cells, and mouse As4.1 cells. An enhancer element was discovered far upstream from the transcription start site (−5777 to −5552). It conferred an ~59-fold activation of transcription in chorionic cells and gave smaller but significant increases in Calu-6 cells (~6-fold) and As4.1 cells (~6.5-fold). When used with a heterologous promoter, enhancer transcriptional activity was selectively increased in renin-producing cells. DNase I footprinting experiments detected at least three specific DNA-protein interactions with chorionic cell nuclear extracts, the functional activity of which was assayed in the three cell types either by reconstitution experiments with oligonucleotides encoding each protected sequence or by site-directed mutagenesis.

This is the first report of a human renin gene enhancer with a chorionic cell-marked effect, suggesting its involvement in the regulation of the transcription of the renin gene in the chorion and that it may be responsible for the expression of the renin gene in different tissues.
EXPERIMENTAL PROCEDURES

Chorionic Cell-specific Human Renin Gene Enhancer

Plasmid Constructs—The pGL3-892 and pGL3-2824 plasmids have been described elsewhere (14). Human renin gene 5′-flanking regions were isolated from a pWE15 cosmid (Stratagene) containing the human renin gene and were inserted into pGL3-892. The fragment (−5777 to −892) was inserted in the sense orientation into the HindIII site of pGL3-892, creating pGL3-5777. Plasmids pGL-4713 and pGL-5339 were created by digesting pGL-5777 with BglII and XmnI, respectively. The (−5777 to −4713) fragment was excised from pGL3-5777 by BglII digestion and was inserted in the sense or antisense orientation into pGL3-892, creating pGL3-892 (+−5777 to −4713) sense and antisense (pGL3-892 (+−5339 to −4955) was created by inserting the DraI/SmaI fragment (−5339 to −4955) into pGL3-892. pGL3-892 (+−5552 to −5539) was created by inserting the XmnIDraI fragment (−5552 to −5539) into pGL3-892. Then, pGL3-892 (+−5777 to −5552) was constructed by insertion of the 1340 bp fragment obtained as described above upstream from the SV40 promoter vector digested by XmnI/SmaI. Each construct was sequenced and verified to be correct.

DNase I protected regions were inserted upstream from the SV40 promoter in the pGL3-promoter vector. The sequence of the renin oligonucleotide corresponding to footprint A (−5766 to −5743) was 5′-CTCGAGATCTCCTAAGAATTACAACCCGTTGGGGG-3′, that corresponding to footprint B (−5747 to −5719) was 5′-CTCGAGATCTGGTGGTGTAGGAGTTCATC-3′, that to footprint AB (−5766 to −5719) was 5′-CTCGAGATCTGGATCAGTATCCCGTTGCGGTCAGAGGCTTCTGGATGCGGC-3′, that to footprint C (−5656 to −5615) was 5′-CTCGAGATCTGGATGCGGC-3′, that to footprint ABC was CTCGAGATCTCCTAAGAATTACAACCCGTTGGGGGGTCA-GAGGAAAAATTGAGTGACTGATTGGGGGAAATGAGCAGATCACATATCAATCCTGCTGTC-3′, and that to footprint AB was CTCGAGATCTCCTAAGAATTACAACCCGTTGGGGGTCAGAGGCAAAATGGAGTCAGTCA-3′. These oligonucleotides were flanked by a XhoI and a BglII site that facilitated the orientation of the fragments upstream from the SV40 promoter in the pGL3-promoter vector digested by BglII and XhoI, creating plasmids pGL3-pGL3-promoter+A, pGL3-promoter+B, pGL3-promoter+AB, and pGL3-promoter+C, and pGL3-promoter+ABC. Each construct was verified by sequencing.

Cell Culture and Transfection Analysis—Primary cultures of chorionic cells, 3.4 renin reporter construct and 30 ng of pCH110 were used with 0.92 or 3.4 respectively. The (−5777 to −4713) fragment was inserted into pGL3-892, creating pGL3-892 (+−5777 to −4713) sense and antisense (pGL3-892 (+−5339 to −4955) was created by inserting the DraI/SmaI fragment (−5339 to −4955) into pGL3-892. pGL3-892 (+−5552 to −5539) was created by inserting the XmnIDraI fragment (−5552 to −5539) into pGL3-892. Then, pGL3-892 (+−5777 to −5552) was constructed by insertion of the 1340 bp fragment obtained as described above upstream from the SV40 promoter vector digested by XmnI/SmaI. Each construct was sequenced and verified to be correct.

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Cell Culture and Transfection Analysis—Primary cultures of chorionic cells, 3.4 renin reporter construct and 30 ng of pGL3-promoter vector digested by BglII and XhoI, creating plasmids pGL3-pGL3-promoter+A, pGL3-promoter+B, pGL3-promoter+AB, and pGL3-promoter+C, and pGL3-promoter+ABC. Each construct was verified by sequencing.

Preparation of Nuclear Extracts, DNase I Footprinting, and electromobility Shift Assays—Human chorionic cell and COS-7 cell nuclear extracts were prepared as described previously (17). The radioactive (−5777 to −5552) fragment was used for DNase I footprinting of the renin promoter was obtained by PCR amplification using the following primers: 5′-TGGAGCTTTTCTGGATATGACG-3′ and 5′-AATCCCCTCCCTGATTAAACAGCAGG-3′. These primers were designed by Andrews et al. (18). 5′-TGGAGGAACTGTCTGCAGCACTGGAGCACAGGGCAGGACATCATCAATGGCTCCTGTCACGTCCAGAAATGAGTTCATC-3′ and 5′-CTGATACCAACGTGGAGTCACTGGAG-3′. The double-stranded oligonucleotides were end-labeled with [γ-32P]ATP by T4 DNA polynucleotide kinase. Each binding reaction was performed with 7 μg of human chorionic cell nuclear extracts incubated with 25,000 cpm end-labeled oligonucleotides for 30 min at 4°C in 20 μl of buffer containing 6 mM MgCl2, 0.8 mM EDTA pH 8.0, 60 mM KCl, 30 mM Hepes pH 7.6, 5% Ficoll 400, 1 mM DTT, 175 mM NaCl, 10 mM Hepes, pH 7.9, 12.5% glycerol, 3 μg of poly(dI-dC), and 1 μg of sonicated salmon sperm DNA. When performing electromobility supershift assays, antibodies directed against c-Jun, c-Fos, JunB, fra-2, kindly provided by Dr. Lallemand (France), URA 1644, DNAse I, and 32P-labeled antibodies were provided by Dr. Deveaux (INSERM U 91) were added to the reaction mixture and then incubated for 1 h at 4°C before electrophoresis. The mixture was subjected to electrophoresis in nondenaturing conditions in 5% polyacrylamide gels in 22 mM Tris borate/0.5 M EDTA. For competition experiments, unlabeled oligonucleotides were used at a 50- to 200-fold molar excess.

Site-directed Mutagenesis by Reombinant PCR—To destroy the NF-E2 and AP-1 binding sites in footprint C, two PCR reactions, one using sense and GL3 primer (Promega) and the other using reverse and RV3 primer (Promega) were performed (mutations are indicated in italics and underlined). These two PCR products were mixed and subjected to a new PCR reaction with GL3 and RV3. The overlap containing the sites allowed the correct recombinant DNA to be obtained. The whole fragment which was inserted into pGL3-promoter vector and fully sequenced. The following oligonucleotides were used: NF-E2mut sense, GAAATGAGTCTACATTAC NF-E2mut reverse, AGTAAGGATCCCTCTTCATTTCC; C/A-lmut sense, TCATTACGTCCTGCTCTACCTC; and C/A-lmut reverse, AAGTACGCGAGGTAAAGGA. Double mutants were obtained by the same strategy.

To destroy the AP-1 sequence of the B fragment, B/AP-1mut reverse (GAGGCGTCTAGCTAGCAGACTCATTCTTTTG) and RV3 were used to perform a PCR using pGL3-promoter-enhancer. The PCR product was digested by BglII and SacI and inserted into pGL3-promoter vector. To create AB(892)-pGL3-promoter-enhancer, a PCR reaction using BglII reverse primer (AGTGGAGGAGCTAGCTCATG) and RV3 was performed using pGL3-892. PCR product was digested by BglII and SacI and ligated into pGL3-promoter-enhancer. The remaining digested vector was blunt-ended and self-ligated to create ΔAB-pGL3-promoter-enhancer.

RESULTS

Identification of an Upstream Positive Regulatory Region—Transcriptional activity of the human renin gene distal promoter was performed by transient transfections of various regions, up to position −8876, inserted upstream from a promoterless luciferase gene construct in human renin-expressing chorionic cells. The activity measured was compared with that in human Calu-6 cells and in mouse As4.1 cells (Fig. 1). When the renin gene 5′-flanking region was lengthened from −892 to −4713, promoter activity, expressed as a percentage of pGL3-892 activity, decreased in human cells whereas it increased progressively in mouse As4.1 cells. The promoter region was lengthened to −5777 after position −4713 while a 57-fold higher transcriptional activity in chorionic cells (4412 ± 489) versus (77 ± 6) for pGL3-4713. These results suggest that there is an enhancer between −5777 bp and −4713 bp that confers −57-fold higher transcription rates in human chorionic cells. We also studied more distal fragments to −8876 and found that the (−4776 to −5777) region contained cis-acting regions that were func-
Calu-6 cells, and As4.1 cells. 

renin-expressing chorionic cells, 

tity of various renin promoter-lucifer- 

performed in triplicate wells.

value is the mean ± S.E. of at least three independent transfection experiments performed in triplicate wells.

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Reporter plasmids were transfected into chorionic cells, Calu-6 cells, As4.1 cells that produce renin, and COS-7 cells that do not produce renin. The results are the mean ± S.E. of at least three independent experiments performed in triplicate wells.

| Renin promoter/luciferase constructs | Percentage of pGL3-promoter relative luciferase activity |
|-------------------------------------|--------------------------------------------------------|
|                                    | Chorionic cells | Calu-6 cells | As4.1 cells | COS-7 cells |
|-------------------------------------|----------------|--------------|-------------|-------------|
| pGL3-promoter vector                | 100 ± 11       | 100 ± 5      | 100 ± 2     | 100 ± 2     |
| pGL3-promoter-enhancer             | 5920 ± 637     | 604 ± 133    | 656 ± 36    | 108 ± 7     |
| pGL3-promoter vector+A             | 178 ± 49       | 153 ± 11     | 115 ± 10    | 90 ± 3      |
| pGL3-promoter vector+B             | 240 ± 8        | 160 ± 20     | 97 ± 5      | 97 ± 2      |
| pGL3-promoter vector+C             | 289 ± 10       | 250 ± 55     | 150 ± 10    | 78 ± 7      |
| pGL3-promoter vector+AB            | 362 ± 82       | 490 ± 80     | 180 ± 12    | 115 ± 11    |
| pGL3-promoter vector+ABC           | 315 ± 31       | 234 ± 8      | 344 ± 9     | 110 ± 7     |

Therefore, the activity of the enhancer was cell-specific and promoter-independent. There was no difference in the activity of the pGL3-promoter and pGL3-promoter+(−5777 to −5552) reporter plasmids in COS-7 cells (Table I), suggesting that the effect of the enhancer on the transcription of the human renin gene may be specific to renin-expressing cells.

**Binding of Chorionic Cell Nuclear Extracts to the Human Renin Enhancer**—To further determine the sequences involved in enhancer activity, an in vitro DNase I footprinting assay was performed using chorionic cell nuclear extracts. Three regions were protected by nuclear proteins (−5766 to −5745), (−5743 to −5719), and (−5651 to −5619), named A, B, and C, respectively (Fig. 2). These three footprints were specific to chorionic cells because DNase I footprinting experiments performed with COS-7 cell nuclear extracts showed only one footprint in the B region, slightly shorter than B and named B (Fig. 2, panel III). To detect consensus matches in protected nucleotide sequences, the enhancer sequence was subjected to a screen of consensus matches against a library of transcription factor binding sites (19). This analysis allowed us to identify a putative binding site for NF-E2 (18) and AP-1 (20, 21) in footprint C and a putative binding site for AP-1 in footprints B and D (Fig. 2, panel II).

To analyze the specificity of the DNA-protein interactions, we performed gel shift assays with oligonucleotides spanning the three protected sequences and chorionic cell nuclear extracts. No specific interactions were detected with an oligonucleotide corresponding to footprint A (data not shown). A weak interaction was detected with the oligonucleotide containing the footprint B (−5747 to −5719), which was, however, specific because unlabeled homologous oligonucleotide competed for binding (Fig. 3). This interaction was not competed by a 100-fold molar excess of oligonucleotides corresponding to foot-

|TABLE I|

**Cell specificity of the human renin gene enhancer**

Each value is the mean ± S.E. of at least three independent transfections performed in triplicate wells.

The footprints B (−5747 to −5719) and C (−5651 to −5619) were formed with COS-7 cell nuclear extracts. Three regions were protected by nuclear proteins (−5766 to −5745), (−5743 to −5719), and (−5651 to −5619), named A, B, and C, respectively (Fig. 2, panel II). These three footprints were specific to chorionic cells because DNase I footprinting experiments performed with COS-7 cell nuclear extracts showed only one footprint in the B region, slightly shorter than B and named B (Fig. 2, panel III). To detect consensus matches in protected nucleotide sequences, the enhancer sequence was subjected to a screen of consensus matches against a library of transcription factor binding sites (19). This analysis allowed us to identify a putative binding site for NF-E2 (18) and AP-1 (20, 21) in footprint C and a putative binding site for AP-1 in footprints B and D (Fig. 2, panel II).

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**Fig. 1. Normalized luciferase activity of various renin promoter-luciferase gene constructs transfected in renin-expressing chorionic cells, Calu-6 cells, and As4.1 cells.** Each value is the mean ± S.E. of at least three independent transfection experiments performed in triplicate wells.
prints A (−5766 to −5743) or C (−5656 to −5615) but was competed by a 50- to 100-fold molar excess of the oligonucleotide containing footprint AB (−5766 to −5719). It was not competed by a 100-fold molar excess of oligonucleotides containing consensus recognition sites for AP-1 or NF-E2. A specific interaction was observed with an oligonucleotide corresponding to footprint C containing both NF-E2 and AP-1 consensus binding sites (Fig. 4). It was competed by unlabeled

Fig. 2. DNase I footprinting of the (−5777 to −5552) region of the human renin gene promoter. In Panels I and III, the left parts represent the sequencing ladder. Lanes 1–4 represent the DNase I footprinting of the coding strand of the (−5777 to −5552) region performed without nuclear extracts (lanes 1 and 2, panel I), or with chorionic cell nuclear extracts (lanes 3 and 4, panel I), or COS-7 cell nuclear extracts (lane 3, panel III). Arrows indicate hypersensitive sites. Panel II, sequences A through C (in boxes) of the DNase I-protected regions of the human renin gene promoter in the presence of chorionic cell nuclear extracts. Consensus matches with known transcription factors binding sites are indicated.

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oligonucleotide containing footprint C but not by a 100-fold molar excess of oligonucleotides corresponding to footprints A, B, or AB. In addition, both AP-1 and NF-E2 oligonucleotides competed for binding to the footprint C sequence.

To examine further the interactions at this motif, gel shift assays were performed with labeled oligonucleotides corresponding to bona fide NF-E2 and AP-1 binding sites. Specific interactions also occurred because bindings were competed specifically by molar excesses of both unlabeled AP-1 and NF-E2 oligonucleotides and by the footprint C sequence (Fig. 5). To discriminate between an AP-1 and/or NF-E2 binding activity to the NF-E2 motif, gel shift experiments were conducted with Human Erythroid Leukemia (HEL) cell nuclear extracts and labeled NF-E2 probe, showing two shifted complexes as described by Deveaux et al. (22) (data not shown). Only the upper AP-1 complex was competed by the footprint C sequence, whereas the lower NF-E2 complex was not competed (data not shown), demonstrating that the interaction with the NF-E2 binding site of the footprint C motif is related to AP-1.

Super-shift experiments were performed to characterize further the proteins present in chorionic cell nuclear extracts that bind to motif C. Experiments conducted with antisera directed against c-Fos, c-Jun, JunB, fra-2, and HEL cell nuclear extracts binding to the NF-E2 probe for antisera directed against MafG/K and p45NF-E2 (data not shown). These data suggest that tissue-specific transcription factors expressed in the chorion, but different from typical members of the basic leucine zipper (bZIP) family, are involved in regulating the activity of the enhancer.

Enhancer Functional Analysis—We addressed the relative importance of each binding site by reconstitution experiments where one copy of each footprint motif was inserted upstream from the SV40 promoter in the pGL3-promoter vector. These constructs were transiently transfected into the three renin-expressing cell types and COS-7 cells. None of these fragments had any effect on SV40 promoter activity in COS-7 cells (Table I). In chorionic cells, the activity of pGL3-promoter+c, pGL3-promoter+AB, and pGL3-promoter+ABC constructs were respectively 2-, 2.5-, 3-, and 3-fold greater than that of pGL3-promoter vector. As described in Table I, none of the footprint motifs alone or in combination was responsible for the enhancer activity in any of the cell types studied (chorionic cells, Calu-6 cells, and As4.1 cells). Therefore, to study each footprint motif in its native context, point mutations were performed in each of the identified regions, i.e. the AP-1 motif in the B region (B/AP-1mut), the AP-1 motif in the C region (C/AP-1mut), and the AP-1 part
of the NF-E2 motif in the C region (NF-E2mut) in pGL3-promoter. In chorionic cells, mutations of the C/AP-1 motif or the NF-E2 motif in footprint C are responsible for the loss of 85 and 82% of the enhancer activity (Fig. 6). When both mutations were performed in the same fragment (C/AP-1mut NF-E2mut), enhancer activity was abolished. In addition, mutation in the AP-1 motif of the B region had no effect alone but enhanced the effects of mutations in the C region. In renin-producing Calu-6 and As4.1 cells, a roughly similar pattern could be observed except that the smaller enhancer activity was never abolished in any single or double mutants. As expected, any of the mutations had no effect on pGL3-promoter-vector activity in COS-7 cells (data not shown).

DISCUSSION

Transgenic animal studies (9–11) did not lead to the identification of cis-acting elements directing cell-specific expression of the human renin gene. Functional analysis of the human renin gene proximal promoter identified positive and negative regulatory elements in chorionic cells (6) and Calu-6 cells (14) and a transcriptional silencer specific to renin-producing cells in the first intron (14, 24). From these studies, it has been suggested that there may be cis-acting regions conferring differential and specific renal and extra-renal human renin gene expression located far upstream from the transcription start site, in the distal promoter. Recently, Yan et al. (12) identified an enhancer in the 5'-flanking region of the human gene that is very similar to that of the enhancer-mediating mouse ren-1c gene expression in As4.1 cells (25).

The aim of the present study was to identify the regulatory elements in the distal regions of the human renin gene promoter responsible for renin expression in the chorion. A 225-bp enhancer located between bases −5777 and −5552 of the human renin gene was shown to activate transcription although to different degrees in the three renin-expressing cell models.
described until now. The degree of activation mediated by the enhancer was 57-fold stimulation in chorionic cells and was weaker in Calu-6 cells (5-fold) and As4.1 cells (8-fold). In human cells, regulatory elements were strictly restricted to this 225-bp fragment because no cis-acting regions were detected either downstream, between bases -552 and -2824, or upstream to bases -8876. Species differences may be responsible for the differences in the activity of the (-7476 to -5777) fragment, which activated transcription in mouse As4.1 cells but not in human cells. The enhancer activity of the 225-bp fragment was promoter-independent because it was roughly similar either with the SV40 promoter or the proximal renin

### Percentage of relative pGL3-promoter relative luciferase activity

| Renin promoter enhancer/luciferase constructs | Chorionic cells | Calu-6 cells | As4.1 cells | COS-7 cells |
|-----------------------------------------------|----------------|--------------|-------------|-------------|
| SV40 LUC                                      | 100 ± 11       | 100 ± 5      | 100 ± 2     | 100 ± 2     |
| A B AP-1                                      | 5920 ± 637     | 604 ± 133    | 656 ± 26    | 108 ± 6     |
| A C AP-1                                      | 4226 ± 734     | 534 ± 35     | 503 ± 145   | 101 ± 11    |
| A C AP-1                                      | 833 ± 197      | 189 ± 8      | 244 ± 116   | 111 ± 6     |
| A B AP-1                                      | 326 ± 60       | 281 ± 69     | 154 ± 31    | 110 ± 4     |
| A B AP-1                                      | 1042 ± 34      | 410 ± 15     | 195 ± 5     | 102 ± 8     |
| A B AP-1                                      | 426 ± 8        | 198 ± 7      | 130 ± 2     | 100 ± 1     |
| A B AP-1                                      | 167 ± 20       | 357 ± 70     | 248 ± 72    | 95 ± 15     |

**Fig. 5.** Gel mobility shift analysis with human chorionic cell nuclear extracts and the NF-E2 or the AP-1 binding sites. Double-stranded oligonucleotides containing the consensus binding site for NF-E2 (underlined) 5'-TGGG-GAACCTGTGCTAGTCGACTGGAG-3' or the consensus AP-1 binding site (underlined) 5'-TGGGAACCTGTGCTAGTCGACTGGAG-3' were used as probes. Competition experiments were performed with a 50- to 100-fold molar excess in the NF-E2 panel and 100-fold molar excess in the AP-1 panel, either with homologous DNA or with the (-5656 to -5615) region of the human renin promoter. Specific DNA-protein complexes are indicated by an arrow.

**Fig. 6.** Functional activity of the cis-acting binding sites in the human renin enhancer. Each value is the mean of at least three independent transfection experiments performed in triplicate wells ± S.E.
promoter. The (−5777 to −5552) region acted typically as a classical enhancer in each renin-expressing cell type in both sense and antisense orientations.

DNase I footprinting assays showed that three regions within the 225-bp fragment were protected by chorionic cell nuclear extracts, whereas only one is protected by non-renin-producing COS-7 cell nuclear extracts. A functional analysis of each footprint motif showed that the overall level of stimulation can be accounted for by the addition of the functional effects of each of the protected regions in Calu-6 and As4.1 cells but cannot account for the final 59-fold stimulation of the transcription in chorionic cells. There were no consensus binding sites for known transcription factors in footprint A, but footprints B and C contained a motif similar to the AP-1 binding site that interacts with bZIP factors of the Jun and Fos families of transcription factors (20, 21). Specific interactions with the footprint B motif were only competed by homologous DNA but not by oligonucleotides spanning consensus AP-1 binding sites, showing that the AP-1 consensus binding site of the footprint B motif is not involved. In addition to its characterized AP-1 binding site, footprint C also contains a consensus binding site for NF-E2, a member of the AP-1 superfamily (18). Transcription factor NF-E2 is a heterodimeric protein comprising the erythroid- and megakaryocytic-restricted p45 NF-E2 and the small bZIP p18 (26), also known as MafK present in most, if not all tissues (27) or the recently described MafG (23). Gel shift assays with anti-p45 NF-E2 and anti-MafG/K antisera did not show, either by supershift or binding inhibition, any related binding activity to the footprint C motif. Gel shift assays with antisera directed against c-Fos, c-Jun, JunB, and fra-2 did not block or supershift the motif C chorionic cell nuclear extracts complex. These results strongly suggest that chorionic nucleoproteins binding to the footprint C motif are related but distinct from well characterized AP-1 family members.

The strong enhancer activity observed in chorionic cells seems to be conferred by footprint C since point mutations in the NF-E2 or the bona fide AP-1 binding site strongly reduced enhancer activity. In addition, enhancer activity was disrupted by both mutations in chorionic cells. Nevertheless, reconstitution experiments showed that footprint C alone cannot confer the enhancer activity. Contacts between proteins bound at nonadjacent sites on a short DNA fragment may be facilitated by distortion of the DNA structure that enhance the recruitment of proteins to their DNA targets (28). The transcription factors binding to this region in chorionic cells may affect the architecture assembly and function of the regulatory nucleoprotein complex. Alternatively, this human renin enhancer activity may result from the cooperative binding of transcription factors to their respective cognate sites. A chorionic-specific AP-1-related bZip transcription factor or a chorionic cell-restricted intermediary factor recruited by the binding of transcription factors may be responsible for the 59-fold enhancer activity in renin-producing chorionic cells.

In summary, this study presents a more complete picture than was previously available of the overall spatial arrangement of regulatory sequences responsible for the human renin gene expression. Moreover, although different levels of enhancer activity were observed in all three renin-producing cell models, interactions with tissue-specific trans-acting factors binding to this enhancer may be responsible for the differential transcriptional regulation of the human renin gene in extra-renal and renal tissue. This is the first case that such a sequence has been shown to be efficient and active in chorionic renin-producing cells. Isolation of the transcription factors involved in the regulation of this enhancer should provide impor-

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**Fig. 7.**

*a,* sequence alignment of conserved residues between the (−5777 to −5605) and (−892 to −717) region. The three DNase I-protected regions, A, B, and C, are boxed. Conserved residues are indicated by stacked dots. Broken lines indicate gaps introduced for optimum alignment. *b,* functional activity of the conserved AB region. Footprints AB of the −5777 region were either deleted or exchanged by the AB region of the −892 region in the pGL3-promoter-enhancer plasmid context. Each value is the mean of at least three independent transfection experiments performed in triplicate wells. Error bars represent S.E.
tant new insights into the molecular events responsible for the differential levels of renin gene expression in the chorion and other tissues.

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