Human Renin mRNA Stability Is Increased in Response to cAMP in Calu-6 Cells

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Abstract—The human carcinoma–derived cell line Calu-6 has previously been demonstrated to endogenously express human renin (hREN) mRNA and to markedly increase steady-state hREN mRNA levels (100-fold after 24 hours) in response to analogues of cAMP and postreceptor activators of adenyl cyclase such as forskolin. However, both transfection analysis using hREN promoter-reporter constructs and nuclear run-on experiments suggest that transcriptional activity alone cannot account for this level of induction. We performed primer extension, reverse transcription–polymerase chain reaction, and 3’ rapid amplification of cDNA ends to compare hREN mRNA between unstimulated and forskolin-stimulated cells. We demonstrate that hREN mRNA is identical under both conditions with respect to (1) utilization of the appropriate transcription start site, (2) processing of renin mRNA, and (3) utilization of the proper polyadenylation site and length of the poly-A tail. To address the mechanism of induction caused by cAMP, we used transcriptional inhibition and measured decay of hREN mRNA before and after forskolin or phorbol ester treatment. Experiments with both actinomycin D and 5,6-dichlororibofuranosylbenzimidazole (DRB) showed that forskolin treatment markedly stabilized hREN mRNA in Calu-6 cells. A 2.3-fold increase in hREN mRNA half-life was also observed after treatment of Calu-6 cells with phorbol ester. Experiments with DRB demonstrated a similar robust stabilization of hREN mRNA after forskolin and phorbol ester treatment. These data demonstrate that the induction in hREN mRNA in response to both cAMP and phorbol ester occurs by a mechanism involving a posttranscriptional component. (Hypertension. 1999;33:900-905.)

Key Words: transcription, genetic ▪ posttranscriptional regulation ▪ RNA, messenger ▪ gene expression regulation

Renin is the first and rate-limiting step in the catalytic conversion of angiotensigen to the peptide hormone angiotensin II. Although the physiological importance of renin has been appreciated for some time, the molecular mechanisms regulating expression of the renin gene remain unclear. Most previous studies of renin gene regulation have primarily focused on transcriptional mechanisms with transfection analysis in renin-expressing and -nonexpressing cell lines (reviewed in Reference 1). One limitation to studies of human renin (hREN) gene regulation stems from the paucity of cells lines that express renin endogenously. We previously reported that Calu-6, an immortalized cell line derived from a human pulmonary carcinoma, expresses hREN endogenously, and it remains the only cell line known to stably express hREN mRNA over many passages. Calu-6 hREN mRNA levels are elevated after treatment with analogues of cAMP, such as 8-bromo-cAMP and dibutyryl cAMP, or postreceptor activators of adenyl cyclase, such as forskolin.

The hREN proximal promoter has been repeatedly demonstrated to be extremely weak, driving only minimal reporter gene expression when transfected into a number of renin expressing and -nonexpressing cell types in culture (reviewed in Reference 1). In addition, our experiments indicate that hREN promoter-reporter constructs containing varying amounts of hREN 5’-flanking DNA extending up to ~5 kb either fail to be expressed or confer an inappropriate pattern tissue- and cell-specific expression in transgenic mice (C.D.S. et al, unpublished observations, 1998). Moreover, transfection analysis revealed only minimal transcriptional induction of the hREN promoter in transfected Calu-6 cells stimulated with forskolin, and maximal stimulation required cotransfection with constitutively active cAMP-dependent protein kinase. Other studies reported a 2- to 4-fold induction in hREN transcriptional activity by forskolin that was partially dependent on the presence of the cAMP response element (CRE) and a POU-domain transcription factor–binding site with homology to Pit-1. In addition, the proximal promoter region of the renin gene contains potential activator protein-1 elements, which bind a transcription factor comprising a c-Jun homodimer or a c-Jun/c-Fos heterodimer. The binding activity of activator protein-1 is stimulated by activators of
protein kinase C (PKC), such as phorbol esters, which have been reported to stimulate renin gene expression in chorionic decidual cells.\(^7\)

To formally test whether the forskolin-induced increase in hREN mRNA involves an increase in hREN transcription, nuclear run-on experiments were performed, and no significant increase in transcription rate was observed.\(^3\) It is therefore necessary to resolve the discrepancy between the 100-fold increase in steady-state hREN mRNA induced by forskolin and the lack of transcriptional induction observed in transfection and nuclear run-on experiments. These results suggest that posttranscriptional mechanisms may be involved in the regulation of hREN mRNA. In studies of cultured mouse juxtaglomerular cells, Chen et al.\(^8\) demonstrated that forskolin stimulation increased mouse renin mRNA stability by approximately 3-fold, suggesting the involvement of posttranscriptional mechanisms in human renin gene regulation. In the current study we demonstrate a posttranscriptional component to the induction of hREN mRNA by forskolin.

**Methods**

Calu-6 cells were maintained as previously described.\(^1\) Cells were grown to 95% confluence and treated with either forskolin (10 \(\mu\)mol/L) or phorbol 12-myristate 13-acetate (TPA; 100 nmol/L) for 24 hours before transcriptional inhibition. Actinomycin D (10.0 \(\mu\)mol/L) or phorbol 12-myristate 13-acetate (TPA; 100 nmol/L) for 24 hours previously described.\(^3\) The hREN cRNA antisense probe was labeled with \(\alpha\)-\(\[^{32}\]P\)UTP and purified on a Sephadex G-50 quick-spin column (Boehringer Mannheim Biochemical). Primer extension was performed as previously described by using a gel-purified, 29-base oligonucleotide with the sequence GCCAGCGCAGCACGCTCTCCATCCATC and end-labeled with \(\gamma\)-\([^{32}\]P\)ATP and T4 polynucleotide kinase.\(^9\) Labeled oligonucleotide (50 000 disintegrations per minute) was hybridized with varying amounts of Calu-6 RNA as indicated. 

DNase treatments and reverse transcription–polymerase chain reactions (RT-PCRs) of Calu-6 RNA were conducted as previously described.\(^11\) The primers for PCR were as follows: TCGTGGCCAAGGGAGAAG (intron 4 up); CTGGCAGAAGGGAGAGAGT (intron 4 down); ATCTGGCCCGGACCCCTTG (intron 9 up); GTGCCCCCTCCCTAATCTGTAGG (intron 9 down); CGGCACCCCCCAGCC (exons 3 to 6 up); and GACACCAGCTTTGATGAGG (exons 3 to 6 down). Rapid amplification of cDNA ends (RACE) was used to confirm the poly-A addition site and to measure the length of the poly-A tail as previously described.\(^11\) An oligo-dT–anchor primer was used for the RT reaction. The anchor portion allows for PCR with a gene-specific primer and an anchor-specific primer. The reaction occurs with \(\alpha\)-\([^{32}\]P\)UTP, and 1000 dpm of the resulting cDNA is run on a denaturing polyacrylamide gel. The upstream (gene-specific) oligonucleotide was CT TAGGCTTAGCTCAGAGTGG, the anchor primer was GGTGATCCCGGCG, and the oligo-dT-anchor primer was GGTGTACCGGTTTTTTTTTTT. Quantification of \(3\)' RACE products, Northern blots, and dot blots were performed using a Molecular Dynamics Storm 420 PhosphorImager and the ImageQuant software provided by the manufacturer. mRNA turnover data are presented as mean±SEM after normalization with respect to GAPDH.

Nuclear extracts from Calu-6 cells were prepared as previously described with the following modifications.\(^12\) Cells from 80% confluent monolayer cultures were harvested and washed in 1× PBS. Pelleted cells were resuspended in buffer containing 10 mmol/L HEPES, pH 7.9; 1.5 mmol/L MgCl\(_2\); 10 mmol/L KCl; and 0.5 mmol/L DTT; maintained on ice to swell; and lysed by being rapidly and repeatedly drawn through a 26-gauge needle.\(^4\) After centrifugation, the nuclear and cytoplasmic extracts were separated, the nuclear pellet was washed with 1× PBS, and RNA was purified as described above. Trypan blue staining indicated the absence of nuclei in the cytoplasmic preparation and the presence of abundant nuclei but not whole cells in the nuclear preparation.

**Results**

Calu-6 cells express human renin endogenously at a low basal level; however, with the addition of forskolin, hREN RNA is
induced by 2 orders of magnitude after 24 hours (Figure 1). A similar albeit less marked induction is seen with the addition of the phorbol ester TPA, an activator of the PKC pathway (Figure 1). The TPA response is similar to that seen in primary cultures of JG cells, in which phorbol esters have been shown to stimulate renin mRNA levels independently of forskolin. A longer exposure revealed low-level hREN expression in the untreated lane, and equal loading of the blot is demonstrated by 28S rRNA staining. We previously demonstrated that transcriptional activity of the hREN promoter could not account for the increase in hREN mRNA caused by forskolin. However, given the 100-fold induction of hREN mRNA in response to forskolin and the difficulty in observing both uninduced and induced mRNAs on the same gel, we performed experiments to ensure that both species of hREN mRNA were identical with respect to transcription initiation and mRNA processing.

Primer extension analysis was performed to compare transcription start sites. An antisense primer was designed to hybridize to hREN mRNA 76 nucleotides downstream of the normal transcription initiation site. The identical primer extension product was observed in both the untreated and forskolin-treated cells, indicating utilization of the same major transcription initiation site in both untreated and forskolin-treated cells (Figure 2). We also confirmed appropriate processing of hREN mRNA by performing RT-PCR with primers spanning the entire mRNA (data not shown). Identical RT-PCR products were obtained from RNA isolated from treated and untreated cells, suggesting that both species of hREN mRNA are identical in overall structure.

In addition to the major species of hREN mRNA observed by Northern blot analysis is a higher-molecular-weight band (labeled with an asterisk in Figure 1). To determine whether this represents unprocessed hREN hnRNA, nuclear and cytoplasmic RNAs from untreated or forskolin-treated Calu-6 cells were subjected to Northern blot analysis. This analysis revealed that the slower-migrating band is nucleus-specific, thus supporting the notion that it is unprocessed hnRNA (Figure 3A). Moreover, similar results were obtained in both untreated and forskolin-treated cells. RT-PCR was performed to provide additional support for the identification of the higher-molecular-weight band as unprocessed hREN hnRNA.

**Figure 3.** Cell fractionation of hREN mRNA and RT-PCR confirmation of hREN hnRNA. A, Calu-6 cells were fractionated into nuclear (N) and cytoplasmic (C) fractions, and RNA (10 μg) from each fraction and a corresponding whole-cell (W) RNA were subjected to Northern blot analysis. *Indicates upper band in Figure 1. B, Schematic representation of the hREN gene, showing the positions of primer sets used to amplify introns (closed arrows) and exons (open arrows) to differentiate immature heterogeneous nuclear RNA from mature mRNA. C, Random hexamers were used to reverse transcribe RNA from nuclear (N) and cytoplasmic (C) fractions purified from Calu-6 cells and subsequently subjected to PCR. The primer sets used in the amplification are indicated.
A schematic of the 2 intron-specific primer sets and the 1 exon-specific primer set used in this analysis is shown in Figure 3B. Intronic RNA was clearly identifiable by RT-PCR in whole-cell (not shown) and nuclear RNA preparations but not in cytoplasmic RNA preparations (Figure 3C). As expected, RT-PCR products consistent with mature mRNA were observed in both the nuclei and cytoplasmic preparations.

To address whether posttranscriptional mechanisms play a role in the induction by forskolin, we measured the decay of hREN mRNA after transcriptional inhibition. Preliminary experiments examining the incorporation of tritiated uridine into trichloroacetic acid–precipitable counts revealed that 10 μg/mL actinomycin D and 50 μg/mL DRB inhibited transcription in Calu-6 cells by 95% and 90%, respectively. Decay curves revealed that the half-life of hREN mRNA in the basal state after actinomycin D treatment was 4.2 hours (Figure 4A). Interestingly, there was no indication of hREN mRNA decay in cells pretreated with forskolin for 24 hours (Figure 4B). The half-life of hREN mRNA was likewise attenuated, albeit to a lesser extent, after pretreatment with TPA (Figure 4C). Although actinomycin D is a very efficient transcriptional inhibitor, it exerts its effects by intercalating between GC base pairs and is known to have a stabilizing affect of its own on many mRNAs. Therefore, it was necessary to repeat these studies with a different transcriptional inhibitor that acts through a distinct mechanism. DRB has been reported to prevent transcriptional initiation without an effect on mRNA stability. With the use of DRB, it was determined that the hREN mRNA half-life was 9.6 hours in untreated cells (Figure 5A) and 8.8 hours after TPA pretreatment (Figure 5C). Like actinomycin D, there was no significant decay of hREN mRNA within the 60-hour period after forskolin pretreatment (Figure 5B).

Differences in the poly-A tail length of mRNA have been previously reported to affect its stability. To confirm utilization of the appropriate poly-A site and to measure poly-A tail length, we used 3′ RACE, a modified RT-PCR protocol that includes an oligo-dT RT primer containing an anchor primer overhang (labeled 1 in Figure 6A). An anchor-specific primer (labeled 2) and a gene-specific primer (labeled 3) are used for the subsequent PCR that results in a characteristic

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**Figure 4.** hREN mRNA decay in the presence of actinomycin D. The decay rate of hREN in Calu-6 cells was determined with actinomycin D. Cells were either untreated (A), pretreated for 24 hours with forskolin (B), or pretreated for 24 hours with TPA (C). Cells were exposed to actinomycin D at time 0 and harvested at the indicated time points. hREN mRNA levels were corrected against GAPDH and using a PhosphorImager (n=3). The half-life (t1/2) of hREN is indicated.

**Figure 5.** hREN mRNA decay in the presence of DRB. The decay rate of hREN in Calu-6 cells was determined with DRB. Cells were left untreated (A), pretreated for 24 hours with forskolin (B), or pretreated for 24 hours with TPA (C). Cells were exposed to DRB at time 0 and harvested at the indicated time points. hREN RNA levels were corrected against GAPDH and using a PhosphorImager (n=3). The half-life (t1/2) of each mRNA is indicated.
pattern of multiple bands due to the variable nature of the hybridization of the RT primer to different positions on the poly-A tail. Our data demonstrate a major 3′ RACE product close to 160 bases long. This finding does not reflect an unpolyadenylated hREN mRNA but results from hybridization of the oligo-dT–anchor primer close to the beginning of the poly-A tail, thus indicating that hREN mRNA from both untreated and forskolin-treated cells is utilizing the same and correct poly-A site. Overall, the same banding pattern was seen in the presence or absence of forskolin, suggesting that the poly-A tail length is very similar in both mRNAs (Figure 6B). The most prevalent 3′ RACE products were 360 to 400 bp long, thus indicating a collection of hREN mRNA molecules with a poly-A tail length of 200 to 240 nucleotides. Although a band at 200 bp is absent from the untreated sample, this result was not reproducible.

**Discussion**

hREN mRNA levels are markedly induced in response to forskolin in Calu-6 cells. We have previously demonstrated that this induction is not due entirely to increased transcriptional activity of the hREN promoter but requires an additional mechanism.3 The present study demonstrates that a posttranscriptional mechanism plays an important role in this induction. Moreover, we demonstrate hREN mRNA induction can also be attained, albeit to a lesser extent, with the phorbol ester TPA, which elicits its effects through the PKC pathway, and that a posttranscriptional mechanism is partially responsible for this induction as well.

In untreated Calu-6 cells, hREN RNA decays with a half-life of 1.7 hours when DRB is added as a transcriptional inhibitor or 4.2 hours when actinomycin D is included as an inhibitor. The difference between the inhibitors may in part be due to the stabilizing effects known to be imparted by actinomycin D.13 It should be noted that this difference can be considered negligible when compared with the stability of hREN mRNA after forskolin treatment, where we found no evidence for turnover even after 60 hours of transcriptional inhibition. Therefore, we could not calculate a precise half-life for hREN mRNA under those conditions. Moreover, we noted a slight upward slope in the hREN decay curves under forskolin-treated conditions. This could be explained by either continued transcription in the presence of the inhibitors, because the measured efficiency of transcriptional inhibition was <100%, or, more likely, to our method of quantifying mRNA decay that relied on GAPDH mRNA as an internal standard. Our studies indicate that the turnover of GAPDH mRNA was negligible during the first 12 hours after transcriptional inhibition but that it turned over more rapidly than did hREN mRNA thereafter (data not shown).

The precise mechanisms causing either enhanced RNA stabilization or decay remain unclear. Most information on posttranscriptional regulation has been obtained from an analysis of the iron response element (IRE) of the transferrin receptor and the AUUUA motif present in the 3′ untranslated region (UTR) of certain immediate-early genes such as c-fos.15,16 Clearly, sequences present within mRNA, primarily but not exclusively located within the 3′ UTR, can determine its stability. Sequences such as the AUUUA can confer decreased stability when placed downstream of normally stable messages.17 The hREN mRNA lacks any AUUUA motifs in its 3′ UTR that might account for its relatively long half-life, even under unstimulated conditions. Although a number of secondary structures can theoretically form within the hREN 3′ UTR, it remains unclear whether these are either necessary or sufficient for increasing stability or whether specific RNA binding proteins like those binding to the transferrin receptor IRE are involved.16

In addition to these specific sequences, a number of studies have demonstrated a correlation between poly-A tail length and mRNA stability, implying that the poly-A tract may protect certain mRNAs from rapid degradation.15 Although our data suggest that both stimulated and unstimulated hREN mRNAs utilize the same poly-A site and have similar poly-A tract length, we cannot formally rule out its importance in regulating the stability of the message. Moreover, our data demonstrating use of the same transcription initiation site rule out the possibility of additional sequences in the 5′ UTR. Although 5′ UTRs have yet to be demonstrated to specifically target RNA binding proteins involved in mRNA stability, their effect can be indirect, by changing overall translational efficiency. Some mRNAs become stable while others are...
more prone to degradation when associated with ribosomes.\textsuperscript{15,18} Moreover, sequences located upstream of the 3' UTR have been shown to be important for regulating mRNA stability.\textsuperscript{19}

One aspect of mRNA turnover that has received considerable attention is the phenomenon of nonsense codon–mediated decay (reviewed in Reference 20). This is observed in a number of genes, particularly disease genes, when premature nonsense codon mutations are present in the mRNA. Although the mechanism remains unclear, the end product is the degradation of the mRNA before a truncated protein is generated. Interestingly, this mechanism may be protective in some disease processes, because loss of protein function may be preferable to the accumulation of abnormal protein that may have dominant negative effects. Moreover, it is likely that the process of scanning an mRNA for degradation in this pathway occurs in the nucleus, either during splicing or mRNA transport.

Unfortunately, this still leaves unresolved the mechanism causing increased hREN mRNA after stimulation of the PKA and PKC pathways. Based on other systems, it is likely that some sequence motif(s) present within the mRNA may be the target of an RNA binding protein(s) that may prevent its turnover, either by stabilizing mRNA structure or by inhibiting the action or binding of ribonucleases designed to degrade mRNA. It remains possible that the synthesis of the RNA binding protein(s) may occur by transcriptional induction via the classic cAMP-CRE pathway or that a pre-existing protein may directly or indirectly require the phosphorylating actions of PKA or PKC. Such a pre-existing protein may be labile, because we previously demonstrated that hREN mRNA induction required active translation.\textsuperscript{3} In conclusion, the abundance of an mRNA depends on 4 factors: (1) the rate of transcription to form a primary RNA product, (2) maturation of this initial RNA transcript, (3) transport of the mature mRNA to the cytoplasm, and (4) the rate of its degradation. Combining both transcriptional and posttranscriptional mechanisms may provide a mechanism for tighter control of gene regulation than can be achieved through transcriptional mechanisms alone.

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