Renin mRNA Quantification Using Polymerase Chain Reaction in Cultured Juxtaglomerular Cells

Short-term Effects of cAMP on Renin mRNA and Secretion

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The aim of the present study was to set up a method to quantify renin mRNA levels in mouse renal juxtaglomerular cells, the main physiological site of renin synthesis. Because of the scarcity of the cells, a quantitative polymerase chain reaction had to be developed to measure renin mRNA. Juxtaglomerular cells were isolated and cultured for 2 days under various conditions, and renin mRNA was measured directly from the cytoplasm of the cultured cells without prior RNA purification. An internal standard consisting of a mutated renin mRNA with an insertion of 60 bp was designed to quantify the reaction, ensuring an identical detection and amplification efficiency to the target RNA. Renin mRNA could be precisely quantified between 0.6 and 20 pg, thus allowing its detection in approximately 5000 juxtaglomerular cells. Forskolin, an activator of adenylate cyclase, led to a concentration-dependent maximal threefold increase in renin mRNA in the cultures after 20 hours of incubation. The half-maximal effective dose was 3×10^{-7} mol/L. The effect of forskolin was mimicked by 10^{-5} mol/L isoproterenol, a β-receptor agonist, and by 10^{-5} mol/L isobutylmethylxanthine. A time-course study showed a rapid increase in renin mRNA within 3 hours after forskolin and isoproterenol addition. Renin secretion in the culture medium was measured in parallel and found to be stimulated by both agents. These results show that quantitative polymerase chain reaction is a suitable tool for studying renin gene expression in cultured juxtaglomerular cells. Our findings indicate that cAMP is a potent and fast activator of renin gene transcription and renin secretion in renal juxtaglomerular cells. (*Circ Res.* 1993;73:639-648.)

KEY WORDS • juxtaglomerular cells • renin mRNA • renin secretion • cAMP • quantitative polymerase chain reaction

The aspartyl protease renin is synthesized, stored, and released exocytotically by renal juxtaglomerular (JG) cells, which are metaplastically transformed vascular smooth muscle cells.[1-3]

The release of renin from the JG cells is the rate-limiting step in the generation of angiotensin II, which is a major determinant of blood pressure and intravascular volume. Renin is synthesized as preprorenin. In the endoplasmic reticulum, a signal peptide is cleaved, yielding prorenin, which is enzymatically inactive. Prorenin is then packaged in storage granules, where it is activated to renin by proteolytic cleavage of an N-terminal profragment.[4-9] Therefore, there are several steps of potential control of renin synthesis and secretion. Evidence obtained in vivo suggests that renin synthesis and secretion are concomitantly regulated by a variety of factors, including blood pressure,[10,11] sodium chloride intake,[12-14] sympathetic nervous output, and angiotensin II level.[15]

Exocytosis of renin is influenced by the intracellular levels of calcium, cAMP, and cGMP, as well as protein kinase C activity.[2,16] However, the effects of these second messengers in the regulation of renin synthesis are not well understood. In particular, it is not known how renin synthesis and renin secretion in renal JG cells are related. This lack of knowledge is mainly due to the lack of experimental models in which renin secretion and renin gene expression could be studied together under controlled conditions.

Recently, we have developed a mouse primary JG cell culture model in which renin synthesis and secretion can be studied in parallel. In this model, de novo synthesis and secretion of renin have been shown.[17] Initial findings have indicated that cAMP stimulated both synthesis and secretion of renin in vitro, but it was not possible to distinguish whether the stimulatory effect of cAMP on renin synthesis in isolated JG cells was due to an increase in translational activity of renin mRNA or primarily due to an increase in renin gene transcription. Since JG cells can be isolated only in small numbers from the kidneys and do not proliferate in culture, it was necessary to establish a sensitive and reliable assay that allows quantification of renin mRNA concentration. Because of the low abundance of the cultured cells and therefore of renin mRNA, common methods used to semiquantify gene expression, like Northern blots, RNase protection, or solution hybridization assays, could not be used.

A reverse transcriptase (RT)–polymerase chain reaction (PCR) method was therefore developed for precisely quantifying mouse renin mRNA levels directly from the cytoplasm of approximatively 5000 cultured JG cells. The very low amount of cells required for one single mRNA measurement makes this method suitable for extensive investigation of renin mRNA accumulation in different experimental conditions.

It has been found that the increase in intracellular levels of cAMP in JG cells led to a time- and dose-dependent increase in renin mRNA levels. Forskolin and isoproterenol significantly stimulated renin mRNA levels within 3 hours. In contrast to isoproterenol, forskolin seemed to produce a biphasic stimulation of renin mRNA levels. This rapid effect was then followed by a secondary delayed increase in renin mRNA levels. These findings suggest that cAMP is a potent and fast-operating regulator of renin mRNA levels in renal JG cells.

Materials and Methods

Isolation of Mouse JG Cells

The mouse strain C57Bl6, which carries a single renin gene (Ren-1^c),[18] was used in this study. JG cells were isolated as previously described.[17] In a typical preparation, the kidneys of five male anesthetized mice (age, 4 to 6 weeks) were removed, decapsulated, and minced with a scalpel blade. The minced tissue was incubated with gentle stirring in buffer 1 (mmol/L: NaCl, 130; KCl, 5; CaCl$_2$, 2; MgCl$_2$, 1; glucose, 10; sucrose, 20; and Tris-HCl, 10; pH 7.4) supplemented with 0.25% trypsin and 0.1% collagenase at 37°C for 70 minutes. The material was then filtered through a 22-μm nylon mesh. Single cells passing through the nylon were collected and washed with buffer 1 (at 500g for 10 minutes). The washed single cells (final volume, 4 mL) were mixed with 60 mL of 30% isosmotic Percoll solution, equally distributed in two centrifugation cups, and centrifuged at 27 000g in a JA-20 rotor and Beckman centrifuge for 25 minutes at 4°C. Four cellular bands differing in density were apparent. The band containing the highest specific renin activity (density, 1.07 g/mL) was used for primary culture.

Primary Culture of Isolated Mouse JG Cells

The cells were rid of Percoll by washing once with 50 mL of buffer 1. The cells were then suspended in 3 mL culture medium (RPMI 1640 supplemented with 0.66 U/mL penicillin, 100 μg/mL streptomycin, and 2% fetal calf serum) and distributed in 100-μL aliquots into 96-well plates. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO$_2$ in air.

Experiments were performed after 20 hours of primary culture, unless otherwise indicated. After 20 hours of primary culture, the culture medium was removed, and the cultures were washed once with culture medium. Fresh culture medium, supplemented as described above, was then added along with the drugs to be tested or their respective solvent controls.

Renin Immunostaining

Renin immunostaining was performed in cultures the second day after seeding. The culture medium was removed, and the cells were washed with phosphate-buffered saline (PBS). The cells were fixed with Bouin's solution for 15 minutes. Afterwards, the cells were washed three times for 2 minutes with PBS, and endogenous peroxidase activity was blocked by incubating the cells in 0.5% H$_2$O$_2$ in methanol for 30 minutes at room temperature. The cells were then permeabilized by adding methanol and keeping them at -20°C for 4 minutes. The cells were rehydrated by washing twice for 15 minutes with PBS and then incubated in blocking buffer (PBS supplemented with 1% bovine serum albumin and 10% fetal calf serum) for 1 hour. Subsequently, the cells were incubated with rat antiserum directed against mouse renin[19] at 4°C overnight (final dilution, 1:500 in blocking buffer). After this incubation, the cells were washed five times with PBS, and biotinylated rabbit immunoglobulin G directed against rat immunoglobulin G (mouse adsorbed) (final dilution, 1:500 in blocking buffer) was added for 2 hours at room temperature. The cells were washed twice with PBS buffer, and immunoreactivity was detected by the avidin-biotin-peroxidase complex using diaminobenzidine/H$_2$O$_2$ as a substrate. Control cells were prepared with normal rat serum instead of antirenin serum.

Northern Blot Experiments

For Northern blot experiments, the JG cells from 40 mice were isolated and distributed in two 25-cm^2 culture flasks. After 20 hours of primary culture, forskolin (10^{-5} mol/L) was added to one flask for 20 hours; the other was used as a control. At the end of the experiment, total RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method of Chomczynsky and Sacchi,[20] yielding 13 and 14 μg of total RNA for the forskolin-treated and the control cells, respectively.

Glyoxal-dimethyl sulfoxide (5 μg)–treated RNA was electrophoresed and transferred to Hybond N filters according to the method of Thomas.[21] RNA blots were exposed to UV light, baked for 1 hour at 80°C, and then prehybridized in 50% formamide, 5× standard saline citrate (SSC; 1× SSC consists of 0.15 mol/L NaCl and 0.015 mol/L sodium citrate), 50 mmol/L sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulfate (SDS), 1× Denhardt's solution (0.02% each of Ficoll 400, polyvinylpyrrolidine, and nuclease-free bovine serum albumin), and 250 μg/mL heat-denatured salmon sperm DNA for 24 hours at 42°C. The blots were then hybridized with a [^{32}P]-labeled 1427-bp mouse renin cDNA[4] for 24 hours at 42°C. The filters were washed four times at room temperature with 2× SSC and 0.1% SDS, then twice at 52°C with 0.1× SSC and 0.1% SDS, and finally twice at 55°C with 0.1× SSC and 0.1% SDS.

To normalize Northern blot results, the total RNA blots were dehybridized by heating at 70°C for 2 hours with 0.005 mol/L Tris-HCl (pH 8.0), 0.002 mol/L EDTA (pH 8.0), and 0.1× Denhardt's solution and rehybridized with a 28S rRNA oligonucleotide probe, according to the method of Barbu and Dautry.[22] The advantage of this probe over other probes such as β-actin derives from observations showing that the abundance of 28S rRNA is directly related to the amount of total RNA present in the same blot.

Densitometric scanning of the autoradiograms was performed with a densitometer (model 620 CCD, Bio-Rad Laboratories, Richmond, Calif) coupled with a 1-D analyst (MacIntosh Data).

Measurement of Renin mRNA by Quantitative RT-PCR

Renin mRNA quantification required setting up an RT-PCR method and the construction of an internal standard that could be easily detected. For this purpose, a synthetic RNA containing a 60-bp insertion was designed.

Choice of the primers. To avoid coamplification of genomic DNA coding for renin, two oligonucleotide primers, one spanning the exon 6/exon 7 border and the other located on exon 8 of the renin gene, were chosen, thus amplifying a 194-bp sequence. Sense primer (5'-ATG AAG GGG GTG TCT GTG GGG TC-3') and antisense primer (5'-ATG TCG GGG AGG GTG GGC ACC TG-3') were synthesized with a PCR-mate DNA synthesizer (Applied Biosystems Inc, Foster City, Calif). Both primers were checked for the absence of fortuitous homology to other known sequences in the GenBank data base.

Internal standard preparation. An internal standard was synthesized by in vitro transcription from plasmid pISMR. This plasmid was constructed from clone pRn 1-4[4]: complete renin cDNA 1427 nucleotides in length was isolated using *Pst* I restriction sites and subcloned into the *Pst* I restriction site of Bluescript BS-SK (Stratagene Inc, La Jolla, Calif). Subsequently, a 60-bp linker fragment corresponding to *Hind*III/*Sac* I polylinker derived from BS-SK was inserted into blunt-ended *Bcl* I site renin cDNA (942 nucleotides). The *Bcl* I restriction site is in the amplified renin fragment. Renin cRNA was prepared as a sense strand from the *Sac* I–digested pISMR template using T7 RNA polymerase according to the in vitro transcription protocol (Stratagene). After the reaction, the template was completely digested with RNase-free DNase I. Renin cDNA could not be amplified in the absence of reverse transcriptase, thus confirming that the internal standard only consisted of renin RNA.

RT reaction on cultured cells.

CELL LYSATE PREPARATION. At the end of the incubation experiments, primary cultured cells were placed on ice, the culture medium was removed, and the cultures were washed once with ice-cold PBS buffer. Then, cells were lysed by the addition of 40 μL of an ice-cold buffer containing 2% Nonidet (NP 40), 10 mmol/L Tris-HCl (pH 8), 10 mmol/L NaCl, 3 mmol/L MgCl$_2$, and 0.7% (vol/vol) mercaptoethanol to each culture well and shaking for 10 minutes at 4°C. The cellular lysates of two culture wells were pooled for the determination of renin mRNA. Cell nuclei were removed by centrifugation at 12 000g and 4°C for 3 minutes. Three microliters of the cytoplasmic fraction was immediately used for reverse transcription; the remaining part was stored at −20°C for cellular protein measurement.

RT REACTION. Ten microliters of an ice-cold solution containing 10 pmol of each PCR primer, 1 μg of yeast tRNA, and 5 pg of the internal standard was added to 3 μL of the cytosolic fraction. Samples were heated for 5 minutes at 65°C and then chilled on ice. Then, 10 μL of solution A was added, and the samples were incubated for 1 hour at 37°C. Solution A consisted of 22 μL of a 25 mmol/L solution of deoxyribonucleotides, 45 μL of 5× RT buffer (supplied with the RT kit), 6 μL of bovine serum albumin (20 mg/mL, Boehringer Mannheim

Corp, Indianapolis, Ind), 6 μL of the RNase inhibitor rRNasin (40 000 U/mL, Promega Corp, Madison, Wis), 9 μL of murine–Moloney leukemia virus RT (200 U/μL, GIBCO-BRL), and 20 μL of 0.1 mol/L dithiothreitol. After incubation, the reaction was stopped by heating the samples for 2 minutes at 95°C.

PCR. To 3 μL of the cDNA solution, 2.5 μL of 10× PCR buffer (supplied with Taq polymerase [Boehringer Mannheim]), 1 μL (10 pmol) of each primer, 4.5 μL of a 25 mmol/L MgCl$_2$ solution, 14 μL H$_2$O, and 10 μCi of [α-^{32}P]dCTP were added. Samples were overlayered with mineral oil, denatured at 94°C for 5 minutes, and cooled to 65°C for 5 minutes. After addition of 1 μL of 25 mmol/L deoxyribonucleotide solution and 1.25 U Taq polymerase, 30 PCR cycles consisting of denaturation at 94°C (1 minute), annealing at 60°C (1 minute), and extension at 72°C (30 seconds) were performed. PCR was completed by a final extension step of 10 minutes at 72°C.

After PCR, the amplification products originating from renin mRNA or from the internal standard were separated by polyacrylamide gel electrophoresis. N,N'-Methylene-bis-acrylamide was replaced by dihydroxy-ethylene-bis-acrylamide. After autoradiography, the bands were excised, solubilized in 0.025 mol/L periodic acid at 50°C, and counted in a beta counter. The radioactivity incorporated by renin mRNA was routinely expressed as a percentage of the radioactivity incorporated by the 5 pg of internal standard. Absolute values for renin mRNA were extrapolated from the standard curve generated with the internal standard: a theoretical correction factor of 1.3 accounted for the minor size of renin mRNA (194 bp) compared with the internal standard (254 bp). Absolute values were used only for data expressing the decrease of renin mRNA levels with the duration of JG cells in culture.

For sequence analysis, the amplified DNA fragment of interest (194 bp) was extracted from 2% agarose gel and purified with a Spin-X filter (Costar Corp, Cambridge, Mass). Both strands of DNA were then directly sequenced by the dideoxy termination method using a thermostable polymerase derived from *Thermus aquaticus* (TAQuence kit version 2.0, United States Biochemical Corp, Cleveland, Ohio).

Determination of Renin Secretion

Renin secretion rates were estimated from the measurement of renin activity appearing in the culture medium at different times. Renin activity was determined by its ability to generate angiotensin I from the plasma of bilaterally nephrectomized rats, as described. Angiotensin I was measured by radioimmunoassay.[23]

Measurement of Protein

Cellular protein concentration was determined according to the method of McKnight.[24] Briefly, 20 μL of the cellular lysate was layered on a GFC membrane (Whatman Inc, Clifton, NJ) and precipitated with trichloroacetic acid. Proteins were stained with 0.25% Coomassie blue, 7.5% acetic acid, and 5% methanol in water.

After washing the membrane with 7.5% acetic acid and 5% methanol in water, specific staining was eluted in 80% methanol, and absorbance was read at 590 nm. Bovine serum albumin was used as a standard.
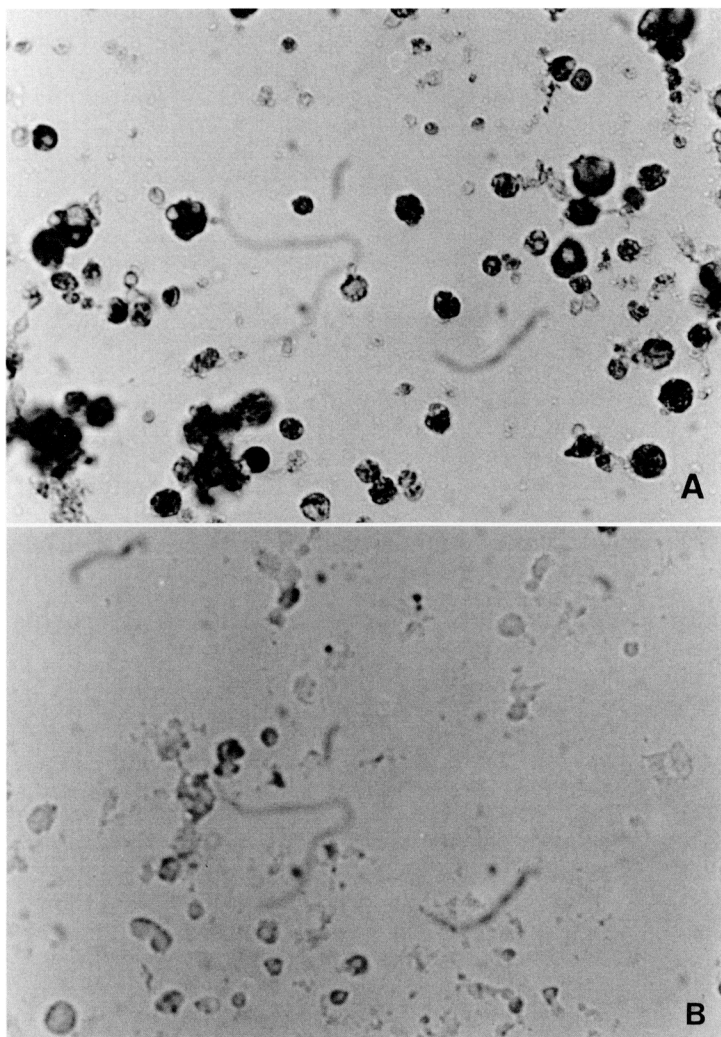
FIG 1. *Photomicrographs show immunoperoxidase staining of mouse juxtaglomerular cells on the second day of primary culture. Specific renin immunoreactivity was observed in 70% to 80% of the cells using a rat anti-mouse renin serum (A). No staining was observed with normal rat serum (B).*

Statistics

Levels of significance were calculated using Student's *t* test. A value of $P<.05$ was considered significant. To compare the time course of renin mRNA and renin stimulation, one way analysis variance (ANOVA) was carried out, and a value of $P<.05$ was considered significant.

Results

Enriched cultures of JG cells were obtained by using a Percoll density gradient separation of single-cell suspensions from mouse kidney. On the second day of primary culture, renin-positive cells represented approximately 70% to 80% of the cultured cells, as assessed by the immunoperoxidase method using a rat antiserum against mouse renin (Fig 1).

In a preliminary experiment, the effect of intracellular cAMP on renin mRNA level in primary cultures of JG cells was determined by Northern blot experiments (Fig 2). The cultured cells were incubated with the receptor-independent activator of adenylate cyclase, forskolin (10^{-5} mol/L), for 20 hours. A single RNA band of 1.4 kb, which had the known size of renal renin mRNA, hybridized with the mouse renin cDNA probe. Densitometric analysis of the autoradiogram demon-strated a twofold increase in renin mRNA by forskolin, as compared with the control value.

However, a single experiment required 40 mice (approximately 1.6×10^7 cells corresponding to 2.25 mg of JG cell protein) to obtain a sufficient amount of renin mRNA; therefore, a more sensitive technique was developed to measure renin mRNA levels.

Detection and Characterization of Renin mRNA by RT-PCR Reaction

A cDNA fragment was amplified after RT reaction and 30 PCR cycles performed directly on the cell lysate of approximatively 5000 JG cells on the second day of primary culture. Four sets of results showed the identity of the amplified product with renin mRNA: (1) The size of the PCR product, resolved by agarose gel electrophoresis and ethidium bromide staining, corresponded to the size of 194 bp predicted for a cDNA originating from renin mRNA (Fig 3A). (2) The amplified cDNA was completely digested by *Sac* I, producing two fragments of 100 and 94 bp (Fig 3A), as expected from the existence of a *Sac* I restriction site located 100 bp 3' of the sense primer in renin cDNA. (3) The amplified DNA hybridized with a ^{32}P-labeled oligonucleotide (5'-GCA TGA TCA ACT TCA GGG AGC TCG TA-3') internal to the amplified mRNA and derived from the
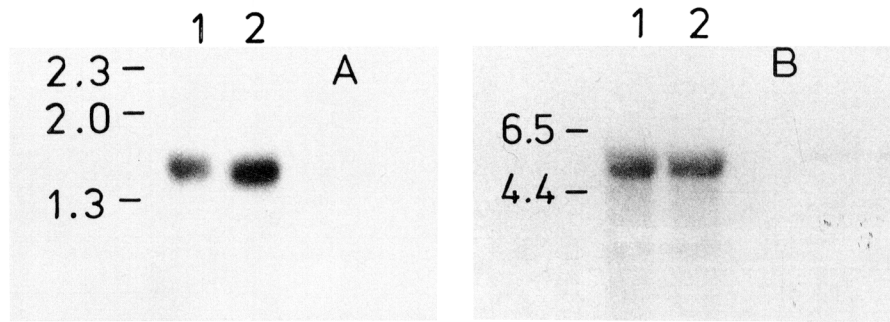
FIG 2. *Renin mRNA expression (A) and 28S RNA signals (B) in primary culture of mouse juxtaglomerular cells. Total RNAs (5 μg per lane) from control cells (1) and forskolin-treated cells (2) were hybridized with a ^{32}P-labeled mouse renin cDNA probe (A). Lanes 1 and 2 were subsequently hybridized with a radiolabeled 28S complementary oligonucleotide probe for normalization (B). The size markers, which are shown in the left margin, are expressed in kilobases.*

mouse renin cDNA sequence (Fig 3B). (4) Direct sequencing of the amplified fragment confirmed the identity that mouse renin mRNA was amplified (not shown).

Quantification of the RT-PCR Reaction

At least 28 PCR cycles were necessary to detect renin mRNA when starting from the cellular lysate of 5000 JG cells on the second day of primary culture. Incorporation of [α-^{32}P]dCTP during the PCR reaction showed that the PCR product was exponentially generated from the 28th to the 32nd cycle (Fig 4). Therefore, all experiments were performed at 30 cycles.

An internal standard consisting of a synthetic renin cRNA with an insertion of 60 bp was constructed to quantify renin mRNA. In an earlier experiment, renin mRNA and the internal standard were amplified separately to investigate whether both were detected with the same efficiency (Fig 5A and 5B). After reverse transcription and 30 PCR cycles, a linear relation between the incorporated radioactivity and the amount of starting material was observed from 0.45 to 15 pg of the internal standard (Fig 5A) and from 0.3 to 10 ng of total RNA extracted from JG cells (Fig 5B). The noninterference of the lysis buffer was studied. The same serial dilutions of total RNA isolated from JG cells and of internal standard were carried out in lysis buffer. The same slope as observed in Fig 5A and 5B with PCR buffer was obtained (not shown).

A standard curve was then generated by plotting the radioactivity incorporated by different amounts of internal standard. To ensure that no competition existed between amplification of internal standard and renin mRNA, serial dilutions of total RNA (from 0.25 to 8 ng) extracted from the JG cells were reverse-transcribed

and coamplified with a fixed amount of internal standard (5 pg). A titration curve was carried out using an increasing amount of internal standard and fixed amount of JG cell mRNA. Ten picograms of internal standard did not affect the amplification of the native mRNA (not shown). The amounts of radioactivity incorporated by renin mRNA were given as the percentage of the radioactivity incorporated by the internal standard and plotted against the total RNA concentration (Fig 5C). The autoradiogram showed that 5 pg of internal standard was not attenuated with increasing amounts of total RNA. No significant difference was observed in the slope of the three dilution curves in Fig 5, showing that internal standard and renin mRNA were amplified with the same efficiency.

On the second day of primary culture, renin mRNA level in the JG cells was 4.70±0.40 pg renin mRNA per microgram cellular protein (n=20). Renin mRNA levels decreased steadily and significantly ($P<.05$) to 17±3% (n=4) and to 7±5% (n=2) of the initial value on the 10th and the 17th day from onset of culture, respectively. From the 30th culture day, no renin mRNA could be detected.

Regulation of Renin mRNA by cAMP

The effect of cAMP on renin mRNA in isolated JG cells was examined by different experiments: (1) a receptor-independent activator of adenylate cyclase (forskolin from 3×10^{-8} to 10^{-5} mol/L) and isoproterenol (10^{-5} mol/L), which stimulated the cyclase by β-adrenergic receptor interaction, and (2) the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 10^{-5} mol/L). In a typical experiment, the JG cells of five mice were isolated and distributed into 30 culture wells. On the second day of primary culture, the cellular
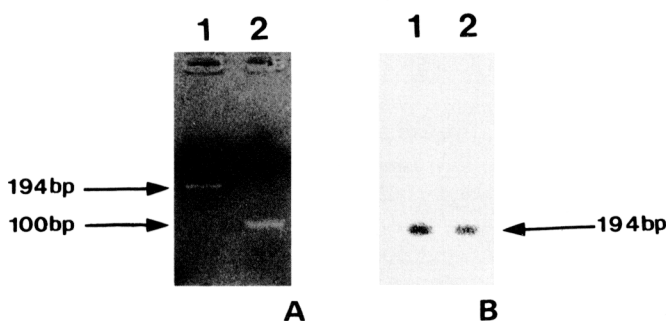

FIG 3. *Characterization of the polymerase chain reaction (PCR) product. A shows ethidium bromide staining of the PCR product before (lane 1) and after (lane 2) digestion by* Sac I. *The samples were electrophoresed on a 2% agarose gel. B shows hybridization of the PCR product with a ^{32}P-labeled oligonucleotide derived from exon 7 of the mouse renin gene. Reverse transcriptase PCR was performed on total RNA extracted from the juxtaglomerular cells (lane 1) and directly on the cytoplasm of juxtaglomerular cells (lane 2) on the second day of primary culture.*
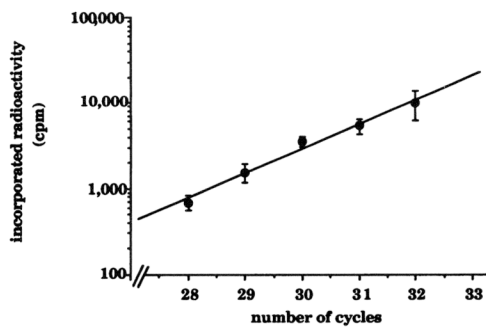
FIG 4. *Analysis of the amplification conditions. Renin cDNA was obtained by reverse transcription of renin RNA from 5000 cultured juxtaglomerular cells. The radioactivity incorporated by renin mRNA was plotted against the number of amplification cycles. Data are mean±SEM (n=4).*

FIG 6. *Bar graph shows regulation of renin mRNA by cAMP. The juxtaglomerular cells were incubated for 20 hours with 10^{-5} mol/L of forskolin (n=11), isoproterenol (n=9), or 3-isobutyl-1-methylxanthine (IBMX, n=4). Values were expressed as percentage of the control. Data are mean±SEM. On the ordinate, 100% corresponded to 4.7±0.40 pg renin mRNA per 5000 cells (n=20).*

protein content averaged 9 μg for each culture well. After lysis of the cells and separation of the cell nuclei by centrifugation, mRNA was reverse-transcribed and PCR-amplified. A specific amplification product was detected from an aliquot of each lysate corresponding to 5000 cultured cells.

As shown in Fig 6, forskolin, isoproterenol, and IBMX caused statistically significant increases in renin
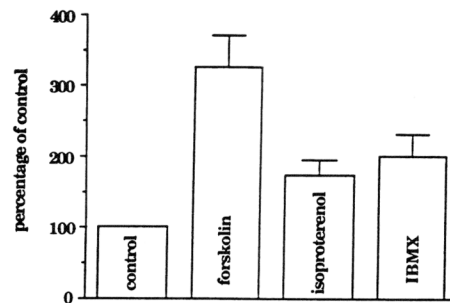
mRNA levels in the cultured cells after 20 hours of incubation ($P<.05$). The strongest stimulation of renin mRNA was observed with forskolin. To determine the maximal stimulation of renin mRNA by cAMP, the concentration dependence of the effect of forskolin on renin mRNA was examined. As shown in Fig 7, forskolin produced a clear dose-related effect on the renin
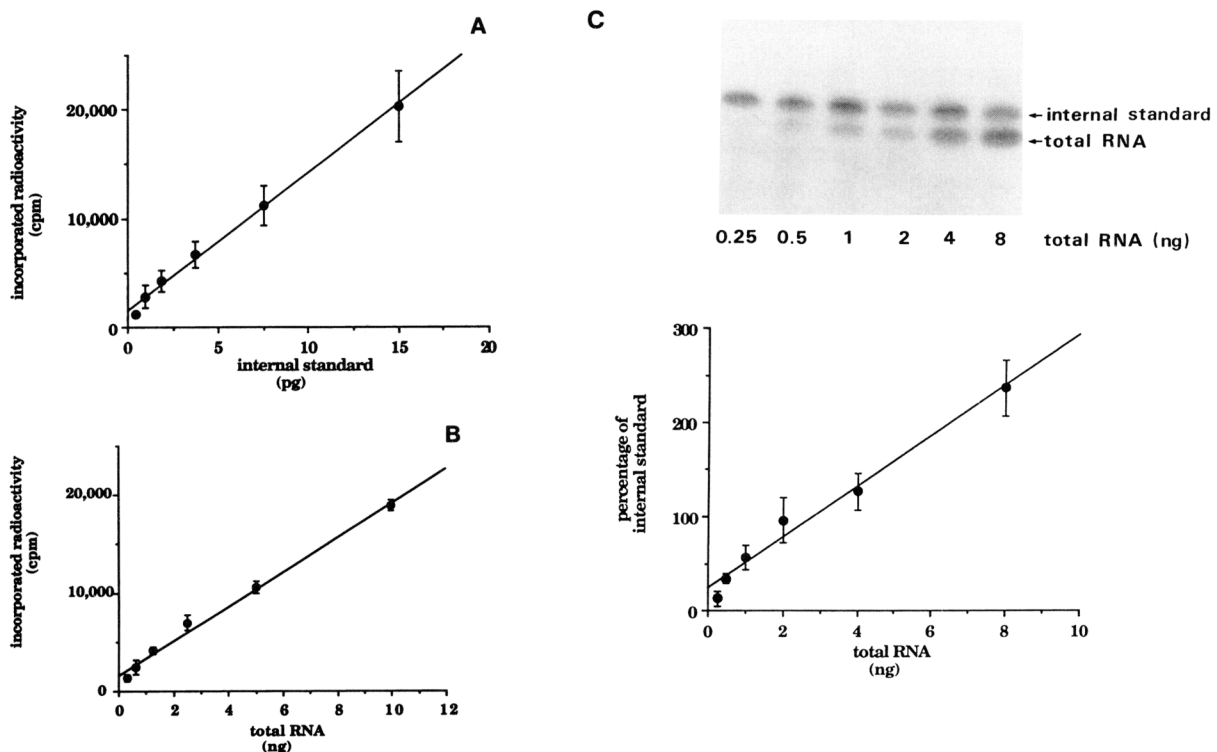

FIG 5. *Quantitative analysis of renin mRNA levels from cultured juxtaglomerular cells. A, Increasing concentrations (0.45 to 15 pg) of the internal standard were plotted against the radioactivity of their polymerase chain reaction (PCR) products. Data are mean±SEM (n=4). B, Increasing concentrations (0.3 to 10 ng) of total RNA extracted from juxtaglomerular cells were plotted against the radioactivity of their PCR products. Data are mean±SEM (n=5). C, Increasing concentrations (0.25 to 8 ng) of total RNA extracted from juxtaglomerular cells were coamplified with 5 pg of the internal standard. At the top is an autoradiogram of a single experiment. In the graph at the bottom, values were expressed as percentage of the radioactivity incorporated by the internal standard and plotted against total RNA concentration. Absolute values for renin mRNA were then extrapolated from this curve and corrected by a factor of 1.3 because of the minor size of renin mRNA compared with the internal standard. Data are mean±SEM (n=4).*
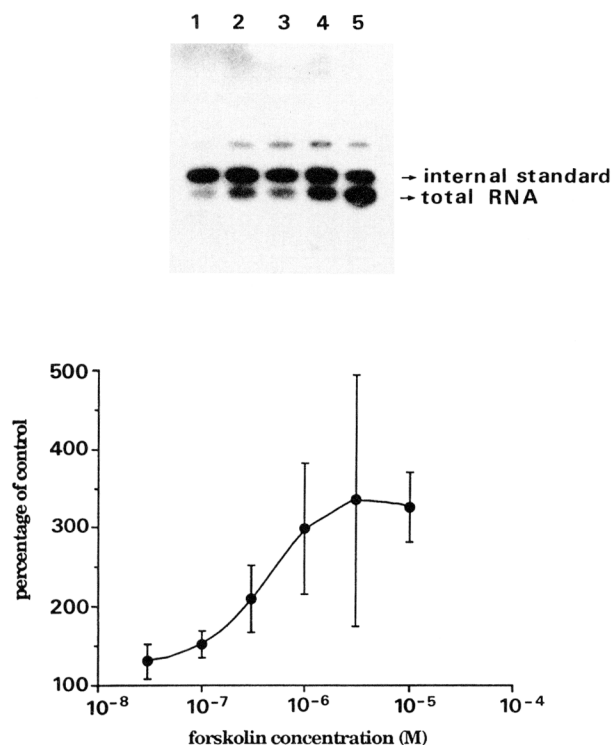
FIG 7. *Dose-response effect of forskolin on renin mRNA levels during 20 hours of incubation. At the top is an autoradiogram of a typical experiment (lane 1, control; lanes 2 through 5, 3×10^{-8}, 10^{-7}, 3×10^{-7}, and 10^{-6} mol/L forskolin, respectively). Although the sense polymerase chain reaction primer was chosen to be cDNA specific, amplification of genomic DNA originating from the hybridization of 15 nucleotides of the sense primer with exon 7 of renin DNA was occasionally observed, probably as a consequence of the partial lysis of cell nuclei during the preparation of cytoplasm, but amplification of genomic DNA (454 bp) was much less than amplification of renin mRNA (194 bp), and their size difference allowed easy identification. At the bottom are renin mRNA levels plotted against different forskolin concentrations. Values were expressed as percentage of the control value. Data are means of 2 different experiments for 3×10^{-8}, 3×10^{-7}, and 3×10^{-6} mol/L forskolin, 4 different experiments for 10^{-7} mol/L forskolin, 10 different experiments for 10^{-6} mol/L forskolin, and 11 different experiments for 10^{-5} mol/L forskolin. Bars represent the SEM in case more than 2 experiments were carried out. On the ordinate, 100% corresponded to 4.7 ± 0.40 pg renin mRNA per 5000 cells (n=20).*

mRNA level, which was maximally increased by 200% over the basal level at 10^{-5} mol/L (10^{-5}, 10^{-6}, and 3×10^{-7} mol/L of forskolin were statistically significant at $P<.05$). ED_{50} for this effect was 3×10^{-7} mol/L.

A final set of experiments was performed to assess the time dependence of forskolin, isoproterenol, and IBMX effects on renin mRNA levels and also on renin secretion in the cultured JG cells (Fig 8). Two-factor ANOVA showed that forskolin (Fig 8A) increased renin mRNA levels (F, 7.8; $P<.001$) and renin secretion (F, 40; $P<.001$) to a higher extent than did isoproterenol (Fig 8B). Forskolin seemed to produce a biphasic increase in renin mRNA. Values of renin mRNA stimulation by forskolin were statistically different for each time of incubation tested (F, 3.05; $P<.05$). A rapid and significant elevation of renin mRNA levels was present at 3 and 6 hours of incubation ($P<.05$). This effect was then followed by a secondary delayed increase in mRNA levels at 20 hours ($P<.05$). For isoproterenol, a significant elevation of renin mRNA levels compared with the control appeared within 3 hours of incubation ($P<.05$) until 20 hours of incubation ($P<.05$). The same level of stimulation was observed for isoproterenol during the time course. For IBMX (data not shown), a significant elevation of renin mRNA levels compared with the control value appeared after 6 hours of incubation ($P<.05$). The level of renin mRNA stimulation was significantly higher at 16 and 20 hours of incubation compared with 1 and 3 hours ($P<.05$). The renin secretion rate was increased and statistically different for each time of incubation by forskolin (F, 29.5; $P<.001$), isoproterenol (F, 27.9; $P<.001$), and IBMX (F, 4.84; $P<.05$). A rapid and significant secretion of renin was observed during the first 3 and 6 hours of culture for forskolin and isoproterenol, respectively ($P<.05$). This was followed by a much slower secretion rate (approximatively two times less) before a secondary increase for both forskolin and isoproterenol between 12 and 20 hours of incubation ($P<.05$).

Discussion

The aim of the present study was to investigate the effect of cAMP on renin secretion and renin gene transcription in renal JG cells by measuring small amounts of renin mRNA. The model chosen was primary cultures of mouse kidney cells that were highly enriched in JG cells. These cells, which represent 0.01% to 0.1% of the kidney cells,[3] can be isolated only in small numbers and do not proliferate under in vitro conditions. However, they store renin and can regulate renin secretion in response to various stimuli such as variations in intracellular levels of cAMP or cGMP.[16] They can also synthesize renin, as recently shown by immunoprecipitation of specific newly synthesized [^{35}S]methionine–labeled renin.[17] Forskolin and isoproterenol appeared to be potent stimulators of renin synthesis in this model.[17]

To further explore the mechanisms leading to renin synthesis, evaluation of renin mRNA accumulation is necessary. Common methods for measuring mRNA levels, eg, Northern blots and RNAse protection or solution hybridization assays, have been used to semiquantify mRNAs but require micrograms of total RNA and are therefore not suitable for large-scale investigations of renin gene expression in cultured JG cells. Indeed, the JG cells of at least 20 mice were required, in the present study, for a single Northern blot experiment. Moreover, to quantify differences in Northern blot experiments, we have used densitometric analysis, which is limited by the saturation of autoradiographed film.

In recent years, PCR has been shown to be an extremely sensitive tool to detect low levels of mRNA.[25,26] It also allows precise and absolute quantification of specific mRNA when an appropriate internal standard is used.[27] PCR has already been used to detect the presence of renin mRNA in the single JG apparatus of the rabbit[28] or in extrarenal tissues of mice and rats.[29] In a recent study, Okura et al[30] reported a method based on PCR to measure rat renin mRNA in extrare-
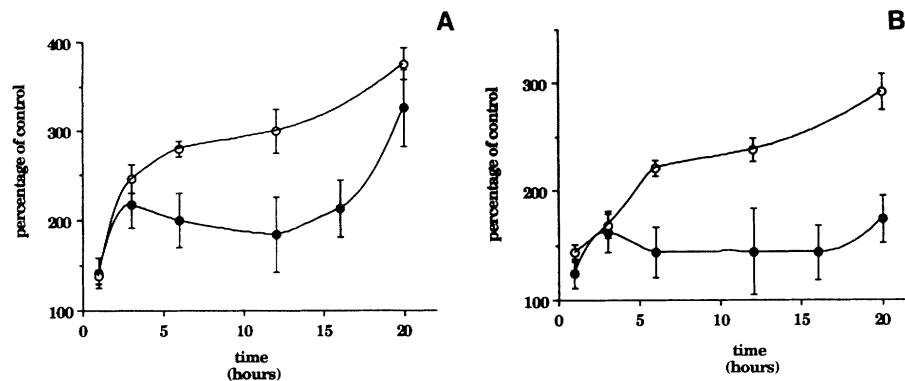
FIG 8.   *Graphs show renin mRNA levels (●) and renin secretion (○) from cultured juxtaglomerular cells after the addition of 10^{-5} mol/L forskolin (A) and 10^{-5} mol/L isoproterenol (B). Renin mRNA data for forskolin represent the mean, and the bars represent the SEM of 3 experiments for 12 and 16 hours of incubation, 5 experiments for 1 hour of incubation, 7 experiments for 3 and 6 hours of incubation, and 11 experiments for 20 hours of incubation. Renin mRNA data for isoproterenol represent the mean, and the bars represent the SEM of 3 experiments for 12 and 16 hours incubation, 4 experiments for 1, 3, and 6 hours of incubation, and 9 experiments for 20 hours of incubation. Renin secretion data represent the mean, and the bars represent the SEM of 3 experiments for both forskolin and isoproterenol. At the onset of the experiment, control renin mRNA levels averaged 9 ± 1.3 pg RNA per 5000 cells (n=4) and spontaneously decreased to 4.9 ± 0.3 pg per 5000 cells (n=9) after 20 hours. After 1 hour, released renin activity was 0.93 ± 0.04 ng angiotensin I per hour per microgram cell protein (n=3) and spontaneously increased to 1.29 ± 0.05 ng angiotensin I per hour per microgram cell protein (n=3). Control values were set to 100% at each time.*

nal tissues. Different levels of renin mRNA were found in different organs, but precise quantification could not be performed because of the lack of an internal standard. To ensure identical detection and amplification efficiency, the internal standard should ideally be reverse-transcribed together with the endogenous mRNA and coamplified and should resemble as much as possible the native mRNA. Recently, a competitive PCR method was described by Iwai and Inagami[31] to quantify rat renin mRNA in extrarenal tissues. In this study, renin mRNA was quantified by amplifying the RNA of interest with different amounts of an internal standard.

A quantitative PCR method, based on the data reported by Wang et al[27] to quantify several lymphokine mRNAs, was developed in the present study for renin mRNA. In the present study, a defined amount of a synthetic RNA was coamplified by the same primers as the RNA of interest and used as reference. This method was applied to primary cultures of mouse JG cells.

Optimal amplification conditions for renin mRNA were set up using total RNA extracted from cultured JG cells. To amplify renin mRNA, 1 ng of total RNA was sufficient. RT-PCR detection of renin mRNA was therefore approximately 5000-fold more sensitive than the Northern blot technique. RNA extraction, however, required relatively large amounts of cells: the JG cells of 20 mice yield 10 to 15 μg of total RNA. Therefore, a technique was developed in which the cultured cells were lysed and which allowed renin mRNA detection directly from the cytoplasm of the cells, without prior RNA purification. The very small amount of cells required for a single mRNA measurement makes the method suitable for extensive investigation of renin gene expression in different experimental conditions.

The identity of the amplified product with renin mRNA was demonstrated by its correct size, by its complete digestion by *Sac* I, as predicted, and by its hybridization with an oligonucleotide derived from the mouse renin cDNA sequence. Messenger RNA quanti-

fication was achieved by the use of an internal standard added to each sample before the RT reaction. To normalize differences among the different experimental conditions, renin mRNA levels were routinely expressed as percentages of the internal standard. Serial dilutions of renin mRNA and of the internal standard confirmed that they were both detected and amplified with the same efficiency. By using the externally generated standard curve, absolute values for renin mRNA could be extrapolated. Absolute quantification of renin mRNA was important because the sensitivity of the PCR method allows detection of minimal amounts of RNAs in many cell types whose physiological relevance is questionable.[32] Absolute quantification of renin mRNA in isolated JG cells allows the comparison of the present results with those obtained in other experiments or in other laboratories. A linear relation between the incorporated radioactivity and the initial amount of RNA was observed in the range from 0.25 to 8 ng for total RNA and from 0.45 to 15 pg for the internal standard. As estimated from Fig 5C, renin mRNA represented approximately 2000 pg renin mRNA per microgram of total RNA present in the JG cells at the second culture day, ie, 0.2% of total RNA. This value represents a 400-fold enhancement compared with the 5.2 pg per microgram RNA (0.0005%) estimated for mouse total kidney RNA by Paul et al,[33] who used a solution hybridization assay, and may correspond to the enrichment in renin synthesizing cells in this cell culture. The dilution curves were used to optimize the initial quantity of cellular lysate and internal standard to avoid substrate depletions, which would impair quantification. This method was then applied to primary cultures of mouse JG cells.

Our findings showed that conditions increasing intracellular cAMP concentration, such as receptor-independent activation of adenylate cyclase by forskolin, β-adrenergic receptor stimulation by isoproterenol, and inhibition of cAMP degradation by IBMX, clearly stim-

ulated renin mRNA levels by a factor of 2 to 3 after 20 hours of incubation.

The quantification of renin mRNA by PCR was consistent with the semiquantitative result obtained by Northern blot, where forskolin (10^{-5} mol/L) stimulated renin mRNA by a factor of 2 after 20 hours. These results are also in agreement with the data recently reported by Rayson[34] and Everett et al,[35] who used Northern blot techniques and estimated a 5- and a 1.6-fold increase of renin mRNA levels in response to increased cAMP levels in rat JG cells cultured on a basement membrane and in rat renal microvessels, respectively. The quantitative PCR technique allowed the construction of a dose-response curve. Forskolin produced a clear dose-related effect on renin mRNA levels, reaching a maximal threefold stimulation between 3×10^{-6} and 10^{-5} mol/L, with an estimated ED_{50} of 3×10^{-7} mol/L. A similar dose-response pattern to forskolin has been reported for renin secretion in rabbit renal cortical slices[36] and for prorenin secretion in cultured human transfected JG cells.[37] The ED_{50} value of 3×10^{-7} mol/L obtained for forskolin on renin mRNA levels in the present study is similar to the ED_{50} of 5×10^{-7} mol/L reported by Pinet et al[37] for forskolin on cAMP levels in human transfected JG cells.

By use of quantitative PCR, it was possible to compare for the first time the time course of the forskolin-, isoproterenol-, and IBMX-induced increases in renin mRNA and renin secretion in cultured JG cells. Renin mRNA levels were increased within 1 hour after forskolin or isoproterenol addition and became significant after 3 hours. Response to IBMX was delayed because increase in renin mRNA levels became significant after 6 hours. In contrast to isoproterenol and IBMX, forskolin seemed to produce a biphasic increase in renin mRNA levels, which reached a maximum after 3 hours. A secondary delayed increase in renin mRNA was observed between 15 and 20 hours.

This biphasic profile of hormone mRNA expression is also found in other secretory cell types. For example, after acute forskolin treatment, a strikingly similar time-course profile has been described for the somatostatin mRNA increase in rat pancreatic islet cells[38]: after a rapid stimulatory phase, a secondary delayed but more potent increase of somatostatin mRNA was observed. The authors explained this rapid rise by cAMP-mediated stimulation of somatostatin gene transcription via protein kinase A–dependent activation of cAMP responsive element–binding protein and suggested the possibility of a delayed stimulation of new protein synthesis as a compensatory response to replenish cellular somatostatin storage pools. It would therefore be interesting to investigate whether increased intracellular cAMP levels are a prerequisite for the second stimulatory phase of renin mRNA levels or whether this secondary phase represents a response to replenish the renin pool after the initial large secretion by a yet-unidentified mechanism. At the same time, renin secretion rates were determined and compared with renin mRNA regulation. The rate of renin secretion was nonlinear, but there was no clear existence of a biphasic secretion in response to cAMP. Whereas renin mRNA levels plateaued after 3 hours before a subsequent increase at approximatively 12 hours, an acute rise in renin secretion was observed until 5 to 6 hours after

forskolin addition. This rapid stimulation was then followed by a slower steady increase lasting at least 20 hours. The stimulatory profile of isoproterenol on renin mRNA levels and renin secretion was similar to that observed for forskolin.

The comparison of the present results with our previous reported data, showing a threefold increase in renin synthesis over 20 hours of forskolin action,[17] suggests that, at the level of JG cells, changes in renin synthesis rate reflect changes in renin mRNA levels. In agreement with this interpretation is the parallelism between our results, showing a maximal threefold increase in renin mRNA levels, and those of Nakamura et al[39] and Horiuchi et al,[40] showing a 2.5- to 3-fold increase in the promoter activity of the mouse Ren-1^d renin gene in response to cAMP.

Taken together, our findings suggest that cAMP is a potent and fast-operating stimulator of renin mRNA levels and renin secretion rates in cultured renal JG cells. Renin mRNA levels and renin secretion were rapidly increased in response to short-term stimulations. The different stimulatory profiles suggest, however, the existence of independent regulatory pathways for both processes. Recently, using reverse hemolytic plaque assays, Everett et al[35] observed that the cAMP-mediated stimulation of renin release in developing rat renal microvessels was essentially due to the recruitment of new renin secreting cells. The amplitude of the observed responses in our cell culture model suggests that, in the adult mouse, a similar phenomenon could only partially account for the cAMP-mediated stimulation of renin secretion and renin mRNA levels.

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