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Regulation of the G-protein α_{i-2} Subunit Gene in LLC-PK$_1$ Renal Cells and Isolation of Porcine Genomic Clones Encoding the Gene Promoter*

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Heterotrimeric G-proteins function as signal transducers for a variety of hormone-coupled enzyme and ion transport systems in eukaryotic cells. We have studied G-protein-coupled processes that appear to be developmentally regulated in polarized pig kidney cells (LLC-PK$_1$). Following trypsinization, LLC-PK$_1$ cells differentiate from a rounded cell type to a fully polarized epithelium by 7 days of culture. During this differentiation, the expression of G-protein α_{i-2} subunit mRNA was not detected until day 4 of culture, it peaked at day 6, and declined thereafter. In contrast, G-protein α_s subunit mRNA which peaked on day 4 was easily detected on all culture days. The presence of the α_{i-2} protein on epithelial cell basolateral membranes followed the same pattern of mRNA expression during culture. To understand the developmental expression of the α_{i-2} subunit in non-polarized cells and its potential regulation by hormones and second messengers in polarized cells at the transcriptional level, genomic DNA segments encoding the α_{i-2} gene promoter were isolated from an EMBL-3 porcine genomic library. S$_1$ nuclease analysis of LLC-PK$_1$ mRNA with cRNA probes derived from these DNA segments revealed major and a minor transcriptional start sites 131 and 171 base pairs upstream of the translation initiation site. The porcine and human α_{i-2} subunit genes shared a 78% sequence identity in their 5′ flanks which suggested an evolutionary conservation of *cis* elements required to influence their transcription. The porcine α_{i-2} gene promoter was identified by fusing DNA segments encoding putative 5′-flanking areas of the gene to a plasmid that contained a firefly luciferase reporter gene but lacked a promoter. The minimal promoter was found between −130 and −60 base pairs from the major transcription start site. No typical "TATA-like" sequences were found. However, a "GC" box and a "TGTGG" sequence were two potential *cis* elements required for basal transcription of the porcine gene promoter which shared a 76% sequence identity to the promoter of another GTP-binding protein, the human c-Ha-*ras* proto-oncogene. Transcription of the gene was inhibited following treatment of renal cells with 10^{-8} M dexamethasone. These studies suggest that α_{i-2} gene expression is regulated in LLC-PK$_1$ cells. Identification of the gene's promoter and 5′-flanking sequences provide a basis for elucidating "cis-acting" DNA sequences and "trans-acting" protein factors that act in concert to control the transcriptional regulation of the α_{i-2} gene which may modulate G-protein coupled effector responses in vasopressin-sensitive renal epithelia.

Heterotrimeric guanine nucleotide-binding (G) proteins couple a variety of enzyme and ion transport transmembrane signaling processes in mammalian cells (reviewed in Refs. 1–4). These regulatory proteins are composed of individual α, β, and γ subunits that are encoded by a super gene family that has been conserved by eukaryotes throughout evolution (5–7). Agonist hormones are known to couple to the stimulating transducing G-protein, G_s, resulting in hormonal activation of adenylyl cyclase. In contrast, hormones antagonistic to adenylyl cyclase activity are known to couple to pertussis toxin-sensitive proteins, G_i, resulting in attenuation of adenylyl cyclase activity. We have previously described a model for vasopressin-sensitive adenylyl cyclase activation in the pig kidney cell line LLC-PK$_1$ where G_i serves as a tonic inhibitor of vasopressin activation of adenylyl cyclase. Addition of GTP to the membranes of these polarized cells revealed a strong negative effect of G_i on agonist receptor activity which was influenced by the stoichiometric relationship between G-protein α_i and α_s subunits (8). Similar tonic inhibition by G_i is found in the rat adipocyte (9, 10) and frog bladder epithelia membranes (11).

During culture, LLC-PK$_1$ cells differentiate from a rounded cell type to a fully polarized epithelium. Tonic inhibition of adenylyl cyclase by G_i is blunted or absent until these cells achieved polarity.[1] This finding suggested that during development, epithelia may exhibit a differential regulation in the synthesis of G_s and G_i. Changes in α_i and α_s protein and mRNA levels occur in 3T3-L1 fibroblasts (12) U-937 and HL-60 monocytes (13) following their treatment by hormones and second messengers to induce differentiation. Alteration of G protein isoforms during differentiation and during embryonic cardiac development are accompanied by concomitant changes in hormone responsiveness (14–16). In dictyostelium, differentiation is triggered by a cyclic AMP-dependent increase in the transcription of α subunit genes (17). Hormones and lymphokines that modulate transcription in target genes, such as corticosterone, thyroxine, and interleukin 1 also differentially alter α_i and α_s subunit mRNA levels in rat cerebral cortex, rat fat, rat cardiac heart, and human endothelial cells, respectively (18–21).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M57287.

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[1] D. A. Ausiello, unpublished observations.

We have utilized the polarized renal epithelial cells LLC-PK_1 and A6 as models to study G protein-regulated functions in the kidney. These studies indicated that renal epithelia express G_i proteins which are involved in the regulation of hormone-stimulated adenylyl cyclase and Na^+ transport at topographically distinct basolateral and apical membranes, respectively (8, 22). In LLC-PK_1 and A6 cells, the α_{i-3} subunit is found in Golgi and apical membranes where it is responsible for the modulation of an apically located amiloride-sensitive Na^+ channel, whereas the α_{i-2} subunit is found at the basolateral membrane (22–24). Clonal cells lines of LLC-PK_1 cells stably transfected with a chimeric mouse metallothionein I-α_{i-2} gene overexpress α_{i-2} subunits following transcriptional activation by heavy metals. Such overexpression confirmed that α_{i-2} subunits are targeted selectively to the basolateral membrane where they may attenuate vasopressin-stimulated adenylyl cyclase activity in these cells (24).

Thus, in vasopressin-sensitive renal epithelia there is differential topographical localization and expression of pertussis toxin sensitive α_i subunits of G-proteins responsible for signal transduction and ion channel regulation. At the basolateral membrane, where α_{i-2} subunit is localized, there is functional evidence for differential expression of α_i and α_s subunits with consequent modulation of the vasopressin-sensitive adenylyl cyclase. In the present study we demonstrate a differential expression of the mRNAs for α_s and α_{i-2} during the development of epithelial cell polarity, suggesting that the gene encoding the α_{i-2} in the LLC-PK_1 cells is under transcriptional regulation. In addition this gene can be inhibited by dexamethasone. We also demonstrate the expected expression of α_{i-2} subunit to the basolateral membrane of developing cells during polarization.

Genomic clones encoding the human α_{i-2} subunit gene have been recently reported by Itoh *et al.* (25) and Weinstein *et al.* (26). However, the "cis-acting" elements which control the transcriptional regulation of this α subunit gene (or any other mammalian α subunit gene) have not been determined as their promoters have not been defined. Thus, as a first step in understanding the developmental expression of the α_{i-2} subunit LLC-PK_1 cells and its potential transcriptional regulation, we have isolated the species-specific genomic DNA segments encoding the α_{i-2} gene and characterized its promoter in renal epithelial cells utilizing a sensitive firefly luciferase reporter gene assay. These studies provide a basis for elucidating cis-acting DNA sequences and "trans-acting" protein factors that act in concert to control the transcriptional regulation of the α_{i-2} gene during renal epithelial cell differentiation. Furthermore, the influence on α_{i-2} subunit gene transcription of hormones, second messengers, and lymphokines known to alter α_{i-2} expression in other tissues can now be examined in renal epithelia.

EXPERIMENTAL PROCEDURES

Cell Culture

LLC-PK_1 cells, a polarized epithelial cell line derived from pig kidney, were grown as confluent monolayers and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in 5% CO_2 atmosphere as previously described (8). Cells were plated at a density of $1 \times 10^6/10$ cm^2 and were used for the following experiments at days 2 through 10 after plating.

Immunofluorescence

The antibody AS7, which was used to localize the G-protein α_{i-2} subunit, was kindly provided by Allen Spiegel (National Institutes of Health). The antibody was raised against the carboxyl-terminal decapeptide of the transducin α subunit. Although it reacts on immunoblots with the α_{i-1} and α_{i-2} subunits (27), in LLC-PK_1 cells which lack α_{i-1}, we have found that AS7 is specific for the native (nondenatured) α_{i-2} subunit (24).

LLC-PK_1 cells plated on glass coverslips were fixed for immunofluorescent staining on days 1–10. Cells were fixed in 3% paraformaldehyde for 1 h, permeabilized in 0.1% Triton X-100 for 15 min, and then washed with PBS[2] containing 0.5% bovine serum albumin to reduce nonspecific background staining. The cells were incubated for 2 h in AS7 or preimmune rabbit serum at 1:100 dilutions, washed three times in PBS, and then incubated for 1 h with goat anti-rabbit IgG conjugated to Texas red (Kirkegaard and Perry). Cells were washed three times in PBS, then mounted in PBS/glycerol, and viewed in an Olympus BHS photomicroscope equipped for epifluorescence.

RNA Gel Blots

Poly(A)$^+$ RNA from LLC-PK_1 cells was separated by electrophoresis in 1.0% formaldehyde/agarose gels and then transferred to GeneScreenPlus membranes (Du Pont-New England Nuclear). Membranes were prehybridized for 2 h at 42 °C in the presence of 1% sodium dodecyl sulfate, 1 M NaCl, 10% dextran sulfate, and 50% deionized formamide. Hybridization was performed under similar conditions for 24 h in the presence of a canine α_{i-2} cDNA (28) (kindly provided by C. Homcy, Massachusetts General Hospital), a rat α_s cDNA (29) (kindly provided by R. Reed, Johns Hopkins University), or a human 3′-untranslated cDNA actin probe labeled with [α-^{32}P] ATP by priming with random hexamers followed by extension of these primers with the Klenow fragment of DNA polymerase (30). After hybridization, membranes were washed twice for 30 min each in 0.3 M NaCl 0.03 M sodium citrate at 23 °C, then the same buffer with 1% sodium dodecyl sulfate at 65 °C, followed by 15 mM NaCl, 1.5 mM sodium citrate at 65 °C. The membranes were dried and autoradiographed with Kodak XAR film at −80 °C for 6–96 h with or without Cronex lightning plus intensifying screens. Quantification of hybridization signals was performed by densitometry of the autoradiograms with a LKB ultrascan XL enhanced laser densitometer.

Isolation of Porcine Genomic Clones for α_{i-2}

A plasmid containing a full-length rat olfactory α_{i-2} cDNA was generously provided by R. Reed (Johns Hopkins University) (29). Purified full-length *Eco*RI (1748 bp) and 5′ *Eco*RI-*Bgl*I (136 bp) cDNA restriction fragments derived from this plasmid were labeled with [α-^{32}P]dATP by priming with random hexamers as above (30). ^{32}P-Labeled restriction fragments were used to screen an EMBL-3 adult porcine genomic library (Clontech). Approximately 1.5×10^6 recombinant plaques were screened. Plaques hybridizing to both fragments were isolated by limiting dilution and were rescreened until purity. Three positive clones (10–4, 13–2, and 19–1) were obtained and were subjected to restriction endonuclease mapping and Southern blot analysis (31). The purified *Sal*I-liberated inserts from these clones were subcloned into Bluescript IIKS+ (Stratagene) and used for nucleotide sequencing analysis, further restriction enzyme analysis, subcloning, and expression studies as described below.

Nucleic Acid Sequencing

Oligonucleotide primers 17–18 nucleotides in length were synthesized by the phosphoramidite method (32) on an automated Pharmacia LKB Biotechnology Inc. Gene Assembler Plus synthesizer. Double-stranded templates were alkali denatured and ethanol precipitated (33), and sequencing was performed on both strands as previously described (34) except for the substitution of T7 polymerase as the DNA polymerase and of 7-deaza dGTP for dGTP. Overlapping sequences were assembled and analyzed using University of Wisconsin Genetics Group Software (35).

Cellular Transfection Assays

Plasmid Construction—For identification of promoter activity, poLUC (a promoterless plasmid containing a firefly luciferase reporter gene) (a gift of A. Brasier and J. Habener, Massachusetts General Hospital) was utilized as an expression vector. The luciferase cDNA in poLUC was originally derived from pSVOALΔ5′ (36, 37). The *Sal*I-*Eco*RI 5′-flanking sequences from clones 13–2 and 10–4 were subcloned into poLUC as 13–2 LUC and 10–4 LUC, respectively.

[2] The abbreviations used are: PBS, phosphate-buffered saline; bp, base pair(s); kb, kilobase(s); EGTA, [ethylenebis(oxyethylenenitrilo] tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid.

For nested deletions of the α_{i-2} 5′-flanking sequences, *SalI-Eco*RI 5′-flanking sequences from clones 10–4 was digested with *Xma*III to remove the first intron and exon 1 (coding and 23 bp of untranslated sequences). This 5′-flanking fragment was repaired with the Klenow fragment of DNA polymerase I and ligated to the *Sma*I polylinker site of poLUC to generate a new plasmid (10–4 LUC (*Xma*III)). Nested deletions of the 5′-flanking regions in this plasmid were made by exonuclease III-mung bean nuclease digestion (Stratagene) utilizing unique *Aat*II and *Sal*I sites or by excision of discrete sequences with site-specific restriction enzymes. Deletions were confirmed by nucleic acid sequencing.

Transfections—Plasmid constructs were transfected in equimolar amounts into LLC-PK$_1$ cells by the calcium phosphate precipitation method (38). A minimum of nine transfections/construct were performed. Luciferase activity of cells was measured 48 h after transfection as described below. Transfection efficiency was normalized by cotransfection with 2.5 μg of pSV2Apap, a plasmid carrying a human placental alkaline phosphatase reported gene driven by a Rous sarcoma virus promoter (generously provided by T. Kadesch, University of Pennsylvania) (39). Results are expressed as % increase ± S.E. in luciferase activity of constructs *versus* poLUC, normalized for heat-insensitive alkaline phosphatase activity. Data was analyzed by paired Student's *t* test. In additional studies, cells were also transfected with pRShGRα, a eukaryotic expression plasmid containing 3.0 kb of the human glucocorticoid receptor cDNA driven by a Rous sarcoma virus long terminal repeat promoter (generously provided by R. Evans, Salk Institute) (40) and p59 RAT LUC, a plasmid containing the rat angiotensinogen promoter from −59 to +39 bp and three copies of a head to tail GRE-I, glucocorticoid response element fused to poLUC (a gift of a Brasier and J. Habener, Massachusetts General Hospital).

Luciferase and Alkaline Phospatase Assays

Forty-eight h after transfection, LLC-PK$_1$ cells were washed twice in phosphate-buffered saline (without calcium or magnesium) and then lysed by addition of 1.0 ml of buffer A (1% Triton, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM fresh dithiothreitol). Scraped lysates were transferred to Eppendorf microcentrifuge tubes and centrifuged at 10,000 × *g* for 5 min at 4 °C. The supernatants were transferred to fresh Eppendorf tubes and briefly vortexed prior to assay.

Luciferase Assay—100 μl of cell supernatant was added to 360 μl of buffer B (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM potassium phosphate, pH 7.8, 1 mM dithiothreitol, 2 mM ATP) at 23 °C. To start the reactions, 200 μl of buffer C (0.2 mM luciferin dissolved in 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM dithiothreitol) was injected, and light output was measured using a LKB model 1251 luminometer integrating peak luminescence 1–16 s after injection or buffer C (36).

Placental Alkaline Phosphatase Assay—Assays were modified from the method of Henthorn *et al.* (39). 50 μl of cell supernatant were incubated at 65 °C for 30 min (for heat-sensitive alkaline phosphatase inactivation) and then centrifuged at 10,000 × *g* for 5 min. Triplicate assays were performed in 96-well Elisa plates using 200 μl of substrate, which contained 0.95 M diethanolamine, pH 9.85), 0.28 M NaCl, 0.5 mM $MgCl_2$, and 5 mM pNPhP (Sigma no 104 phosphatase substrate). pNPhP was stored at 4 °C in the dark as a 0.1 M stock solution in the above buffer and was diluted just prior to initiating the reaction. Standard assays were typically initiated by the addition of 8 μl of heat-inactivated cell supernatant at 30 °C for 90 min in the dark. Reactions were monitored at 405 nm absorbance by a Multiskan Plus MKII plate reader (Flow Laboratories).

S_1 Nuclease Protection Assays

Assays were modified after the method of Quarless *et al.* (41). A ^{32}P-labeled cRNA probe was made by SP6 RNA polymerase (Riboprobe™ system, Promega) as described by the manufacturer. The DNA template, 10–4 Luc minus, contained the *SalI-Eco*RI fragment of insert 10–4, (which encompassed approximately 983 bp of putative 5′-flanking segment, exon 1, and an early portion of intron A in the porcine α_{i-2} gene) ligated to poLUC in the antisense orientation. 10–4 Luc minus was linearized before the insertion site of the firefly luciferase cDNA by *Eco*RI digestion. Approximately 150 ng of riboprobe (~2 × 10^6 cpm) purified by Sephadex G-50 chromatography was mixed with either 1) 5 μg of mRNA from non-transfected LLC-PK$_1$ cells, 2) 20 μg of yeast tRNA, or 3) 20 μg of total RNA from LLC-PK$_1$ cells transfected with 10–4 LUC. The mixture was lyophilized, resuspended in 80% formamide 0.4 M NaCl, 1 mM EDTA, and 50 mM MOPS, pH 7.0, boiled for 5 min and allowed to hybridize at 65 °C for 3 h. After hybridization, 250 mM NaCl, 50 mM sodium acetate, pH 4.5, 3 mM $ZnSO_4$, and 200 units of S_1 nuclease (Boehringer Mannheim) were added followed by incubation at 45 °C for 30 min. The nucleic acids in this solution were ethanol precipitated and resuspended in 25% formamide 5 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol. These samples and a ^{32}P-end-labeled *Hae*III ladder of φX174 DNA (New England Biolabs) were electrophoresed on a 6% acrylamide, 8 M urea gel.

RESULTS

Identification of Newly Synthesized a_{i-2} mRNA and Protein during Differentiation of LLC-PK$_1$ Cells—During culture, LLC-PK$_1$ cells differentiate from a rounded cell type to a fully polarized epithelium. Our previous studies demonstrated that G_i isoforms for α_{i-2} and α_{i-3} (but not α_{i-1}) are found in polarized LLC-PK$_1$ cells (24). A very low abundance 2.6-kb mRNA species hybridized to a canine α_{i-2} cDNA in these cells. Therefore, to examine the mRNA expression of these α subunits during culture it was necessary to harvest and pool the RNA from 250 dishes (100 mm) from each time point studied due to the sparsity of cells in the early days of culture. Fifteen μg of poly(A)$^+$ RNA from each day of culture were size fractionated on a formaldehyde gel and transferred to a GeneScreenPlus membrane. As seen in Fig. 1, following hybridization of this membrane with a canine α_{i-2} full-length cDNA probe, a 2.6-kb mRNA species was detected following a 72-h radiographic exposure in day 4 cells, which increased by day 6 and fell thereafter. Further exposure of the membrane did not reveal any hybridizable signals at day 2 or 3. The membrane was then stripped and rehybridized with a rat α_s cDNA probe with a specific activity similar to that of the α_{i-2} probe. A 1.7-kb mRNA species corresponding to α_s was easily detected for all culture days after only 7 h of exposure without an intensifying screen. The α_s mRNA increased by day 4 only declining slightly by day 7. All lanes were matched for poly(A)$^+$ RNA as assessed by ethidium bromide staining and hybridization with a human 3′-untranslated cDNA probe for actin (data not shown). These findings are consistent with the transcriptional activation of the α_{i-2} gene in renal LLC-PK$_1$ cells during their differentiation from non-polarized to a polarized phenotype.

In our previous studies the α_{i-2} protein was found in the cytoplasm but was predominantly localized on the basolateral

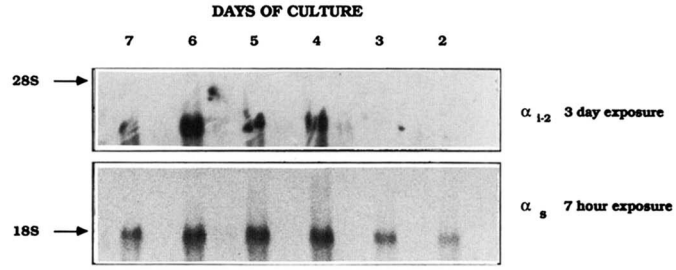

FIG. 1. **RNA gel blot analysis of poly(A)$^+$ from cultures of LLC-PK$_1$ cells harvested from culture days 2 through 7.** A membrane containing 15 μg of poly(A)$^+$ RNA from each culture day was hybridized with a full-length canine α_{i-2} cDNA probe as shown in the *upper panel*. A 2.6-kb mRNA species was detected following a 72-h exposure in day 4 cells, which increased by day 6 and fell thereafter. Further exposure of the membrane did not reveal any hybridizable signals at day 2 or 3. The membrane was then stripped and rehybridized with a rat α_s cDNA probe with a specific activity similar to that of the α_{i-2} probe. The *lower panel* demonstrates the detection of a α_s 1.7-kb species after 7 h of exposure without an intensifying screen. The positions of the 18 S and 28 S ribosomal markers are indicated on the left as determined by ethidium staining of the membrane.

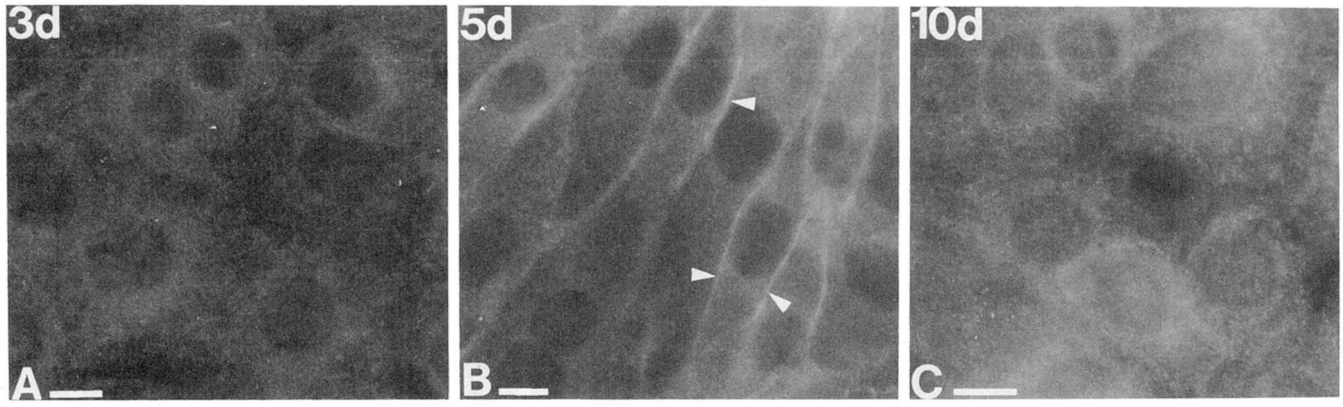

FIG. 2. **Immunofluorescent localization of α_{i-2} in LLC-PK$_1$ cells in cultures at days 3, 5, and 10.** Cell monolayers at days 1–10 were fixed, permeabilized, and stained by immunofluorescence with an anti-peptide antibody which specifically recognized α_{i-2} in these cells. Representative images of cell cultures at days 3, 5, and 10 are shown. *A*, in preconfluent cells from a culture at day 3 (*3d*) only weak cytoplasmic staining can be seen. *B*, at day 5 (*5d*), the cells are confluent and have formed polarized monolayers. Now there is an additional intense staining of the basolateral membranes of these cells. In these monolayers, viewed *en face*, the basolateral membrane staining appears as a single line of fluorescence around the perimeters of each cell (*arrows*). *C*, by day 10 (*10d*) in culture, most of the α_{i-2} basolateral membrane staining has disappeared leaving only diffuse cytoplasmic fluorescence. *Bars* = 10 μm.

membrane of polarized LLC-PK$_1$ cells using immunofluorescent staining (24). Immunoblotting of LLC-PK$_1$ membranes and cytosol confirmed that the α_{i-2} protein was predominantly found in this membrane fraction even after its overexpression in cells stable transfected with a chimeric mouse metalliothionein I-α_{i-2} gene (24). Furthermore, the localization of α_{i-2} subunit has been recently confirmed using confocal image analysis which indicated that this protein colocalized with a known lateral membrane protein, the sodium-potassium ATPase, and was absent from the apical cell surface of LLC-PK$_1$ cells.[3] In cells fixed and stained at Days 1–10 after plating, the presence of the α_{i-2} protein was again found in the cytoplasm, but its appearance on basolateral cell membranes parallelled the expression of α_{i-2} mRNA in these cells. On Days 1–3 there was no detectable basolateral membrane staining of α_{i-2} (Fig. 2*A*), when the cells became fully polarized, days 4–8, α_{i-2} was found predominantly on basolateral membranes which were brightly stained (Fig. 2*B*). On later days (days 9–10), the membrane staining for α_{i-2} began to disappear leaving only cytoplasmic staining (Fig. 2*C*) even though the cells remained fully polarized. Cells from all culture days showed no staining with preimmune serum. Thus, the presence of membrane-associated α_{i-2} protein and the expression of the mRNA coincide at various points during cell differentiation in these LLC-PK$_1$ cultures.

Isolation of Porcine Genomic Clones Encoding the α_{i-2} Gene and Sequence Comparison to the Human α_{i-2} and Other G-protein Genes—One and a half million recombinant phages in a porcine EMBL-3 genomic library were screened with full-length *Eco*RI (1748 bp) and 5′ *Eco*RI-*Bgl*I (136 bp) cDNA rat α_{i-2} cDNA fragments. The inserts of three positives (10–4, 13–2, and 19–1) were subcloned into Bluescript II KS+ for further restriction enzyme mapping and sequence analysis. Each clone encoded a putative exon 1, a variable length of 5′-flanking sequence (10–4, 0.6 kb; 13–2, 11 kb; 19–1, 14 kb) and a first intron (Fig. 3).

Limited sequencing of 5′-flanking area from clone 10–4 revealed a G + C content of 67%, with 42 CpG *versus* 53 GpC dinucleotides and with 64 repetitive elements between 6 to 10 nucleotides in length. No typical "TATA" box was found. However, two "TGTGG" sequences, two "CAAT" boxes, and four GC boxes (sequence CCGCCC or GGGCGG) that could potentially bind a SP1 transcription factor were identified (Fig. 4*A*). One GC box occurred in the noncoding area of exon I, whereas the other three were further upstream (Fig. 4*A*). A consensus cyclic AMP response element for the binding of the cyclic AMP response element-binding protein transcription factor and an AP$_2$-binding consensus sequence was also found (42). Sequences compatible with binding sites of other known DNA transcription factors were not found (43, 44).

The porcine exon 1-coding sequence differed by six and nine conservative nucleotide substitutions from the human genomic and the rat cDNA α_{i-2} sequences which did not alter its amino acid composition. Sequence comparison of the nucleotide sequence of clone 10–4 upstream to the translation start site (Fig. 4) revealed 78.1% identity to the human α_{i-2} subunit gene (374 bp overlap) (24) and 85% identity to the rat olfactory α_{i-2} mRNA (159 bp overlap) (29). Alignment of the porcine and human sequences revealed two deletions of 10 and 12 nucleotides each, at positions 223 and 316 in the porcine gene. The former deletion contained a GC box in the human sequence. A nucleotide substitution at position 570 resulted in the loss of a GC box in the exon 1 non-coding region of the porcine gene. Interestingly, a 54.4% sequence identity was found to the 5′-flanking area of the human c-Ha-*ras* proto-oncogene (522 bp overlap) which encodes a protein that binds and hydrolyzes GTP (45). Furthermore a 38-bp segment (position 353–390) was 76.3% identical to a sequence which occurred immediately upstream of the transcriptional initiation site (position 508–545) in the c-Ha-*ras* proto-oncogene which suggested that the basal transcriptional regulation of these two genes may be similar.

Identification of the Porcine A_{i-2} Transcription Start Site—The transcription start site of porcine α_{i-2} gene was identified by S$_1$ nuclease protection assays. RNA duplexes between LLC-PK$_1$ mRNA and a uniformly ^{32}P-labeled cRNA probe encompassing 995 bases of the 5′ flank and intron A of the α_{i-2} gene were subjected to S$_1$ nuclease digestion. The resulting protected fragments included a major band of 249 nucleotides and a minor band of 285 nucleotides compatible with two transcription start sites in the first exon of the α_{i-2} gene (Fig. 5, *panel A*). Hybridization of this cRNA probe with yeast tRNA followed by S$_1$ nuclease digestion did not produce any

[3] J. L. Stow, manuscript in preparation.

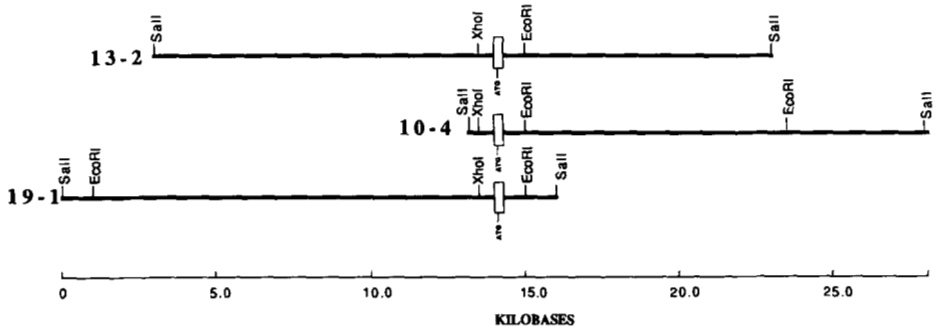

FIG. 3. **Structure and restriction map of the porcine α_{i-2} subunit genomic clones.** Three λ EMBL-3 clones (10–4, 13–2, and 14–1) contained overlapping genomic segments. *Open boxes* indicate the putative position of exon 1 as determined by nucleotide sequencing and Southern blot analysis. Relevant restriction sites for subcloning are shown.

protected fragments (Fig. 5, *panel B*). These findings suggested the α_{i-2} mRNA 5′-untranslated region to be 131 or 168 bases in LLC-PK_1 cells.

Identification of the α_{i-2} Gene Promoter—The plasmid 10–4 LUC (in the sense orientation) was transiently transfected into LLC-PK_1 cells. Forty-eight h later, total RNA was extracted from these cells to determine if the α_{i-2} gene promoter would correctly initiate firefly luciferase mRNA. RNA duplexes between luciferase mRNA and the cRNA probe used above were subjected to S_1 nuclease digestion. Four protected fragments were found. As previously seen, a major band of 249 nucleotides and a minor band of 285 nucleotides were compatible with the two transcription start sites of the native α_{i-2} mRNA (Fig. 5, *panels A* and *C*). Two new protected fragments were also seen, a major band of 531 nucleotides and a minor band of 567 nucleotides that were consistent with the α_{i-2} promoter driven transcription of the firefly luciferase gene (Fig. 5, *panel C*).

To determine the regulatory sequences of the α_{i-2} gene necessary for transcription, the *SalI-EcoRI* inserts of clones 10–4 (983 bp) and 13–2 (11 kb) were ligated in both orientations to poLUC and transiently transfected into LLC-PK_1 cells that were 25–50% confluent and thus would be likely to have maximal transcription of the α_{i-2} gene based on our previous RNA studies. Only transfectants with the 0.6 and the 11 kb of putative 5′-flanking sequence in the sense orientation displayed a significant increase in luciferase activity as compared with cells transfected with the promoterless plasmid (Table I). These data confirmed that the clones 10–4 and 13–2 contain an orientation-specific promoter for the α_{i-2} gene.

Characterization of the α_{i-2} Gene Promoter—To determine the precise location of the α_{i-2} gene promoter, deletion mutants of 10–4 LUC were created. A 3′ deletion of 10–4 LUC (10–4 LUC-(*Xma*III)), which lacked 23 bp of untranslated region, coding region, and the first intron, demonstrated a 53.8-fold increased luciferase activity above poLUC and a 3.2-fold increased luciferase activity above 10–4 LUC. 5′ deletion mutants of (10–4 LUC-(*Xma*III)) were used for all subsequent studies (Fig. 6). Initial 5′ deletions to position −351 (referable to the major transcription start site) reduced luciferase activity to 38-fold above poLUC. An additional fall in luciferase activity to 20-fold above poLUC was seen with a further deletion of 60 bp which contained the most 5′ GC box. Subsequent deletions to position −131 had no effect on luciferase activity although two CAAT boxes were deleted. However, further deletions from −130 to −60 completely abolished luciferase activity to background levels, which indicated the minimal area of the promoter area of this gene.

Successive fine deletions in the promoter area revealed progressive decreases in luciferase activity (i) 12-fold above poLUC after the first 15 bp deletion, (ii) 7-fold above poLUC after an additional 12-bp deletion (which contained the TGTGG sequence), and (iii) 4-fold above poLUC after a further 9-bp deletion into the first base of the second GC box. Further deletions of 35 bp abolished the remaining luciferase activity to background despite the presence of two GC boxes and another TGTGG sequence 3′ to this area.

Dexamethasone Inhibits α_{i-2} Gene Transcription in Renal Epithelial Cells—Previous studies in rodents have demonstrated a reduction in α_{i-2} mRNA levels in cerebral cortex following administration of corticosterone (18). Our initial studies indicated that LLC-PK_1 cells lack sufficient glucocorticoid receptors to induce gene transcription in a steroid-responsive luciferase reported gene plasmid p59 RAT LUC. Steroid-induced gene transcription of this plasmid could be demonstrated in cells simultaneously transfected with pRShGR, a plasmid expression vector encoding the human glucocorticoid receptor (40). Corrected luciferase activity increased from 1,245 units to 32,056 units following treatment of cells with 10^{-8} M dexamethasone. Therefore, to determine whether the α_{i-2} gene was regulated at the level of transcription by steroids in renal epithelia, pRShGRα and the plasmid 13–2 LUC encoding 11 kb of 5′-flanking sequence or poLUC were transiently cotransfected into LLC-PK_1 cells. Transfectants were then treated with or without dexamethasone. As seen in Fig. 7, dexamethasone 10^{-8} M had no effect on the transcription of poLUC, whereas in each of six experiments the transcription of 10–4 LUC was significantly inhibited by 45% ($p < 0.01$). In other experiments concentrations of dexamethasone as high as 5×10^{-6} M had no effect on the transcription of a Rous sarcoma driven promoter fused to luciferase (RSV-LUC) or to transcription of pSV2Apap (data not shown). These data suggested that glucocorticoids may directly inhibit α_{i-2} gene transcription in renal epithelial cells.

DISCUSSION

Previous reports have demonstrated that α_{i-2} mRNA and/or protein content are altered in mammalian tissues both during differentiation and by steroid hormones, lymphokines, or by second messengers such as cyclic AMP. These reports did not determine whether the expression of the gene might be regulated either at the level of transcription, translation, or both. In this report we demonstrate the expression of the G-protein α_{i-2} subunit in renal epithelia during development appears to be under direct transcriptional control. We also provide evidence that α_{i-2} gene transcription can be directly inhibited by glucocorticoids in these cells. The isolation of porcine genomic segments containing 5′-flanking areas of the G-protein α_{i-2} subunit gene and the functional identification of its promoter represent a critical first step in understanding

```
   1 ATCCCAAAACAAGTTTATTGGGTTCATTAACTTTAACAAATGACGAAGAC      a
                     C   AC                     A           b

  51 ACGCCTCCTCTACTCATCTCGCTCGACTCTCAGAAGCTTGGAGGGCTGGT      a
               C  CT A     C A    G        CA               b

 101 TCCTGCTTGGCCCGCGAGGTGCACCGGATCCTCATCCTCGGGCTACGAGA      a
           CT    T     •     •     C C       T  AC          b

 151 TCCGCCCGCCCCCGCCGTCGGGCAGCGGAGCTCCAGGCGCTCCGCACCCA      a
           •T   G  T   CA   A A       C                     b

 201 ATCGGAGCCCGGCCCCGCCTGC••••••••••GATCAAAGCTTCTGTGCT      a
       AC       T         AGCCCGCCCA TG CGG TCC G G T       b

 241 AGGCTCTACCCCTACAGCTATCCCTTGGCGTGGCTCCGCCTTCAGCCCGC      a
     T  AA C       TT  CT TT  CC       C      G  C  A G     b

 291 ACCGAGTTTGGGCTGCGCCTAACTT••••••••••••CTCCCTCGAGCCA      a
           CC      ••          CCCCCTTCGCTC G                b

                                                            c

 329 ATCACTGACCCCGGGTCTCCTGTGGCCCCGCCTCCGGGCCCGCCCCGGCC      a
         ACAG  T TAA      C          G AA                   b
                              *****CG*C**C*C************      c

                                    Minor start site ⇓
 379 CAGTCCTCGGCCAGACTAGCTCGGGCCCCACCCCCGGCCCGCCCTGCCGT      a
         ACA   TT GT C  C A                        C         b
     TC*G**C*****                                           c

                       Major start site ⇓
 429 CGGTGCGCGGCGGCCTGGAAGGCACCGCCTGCAGACGCTCGGAACTGCGG      a
           ↑       TAG        G  T  C    T             C    b
                   G G        G  T  C    T                  d

 479 ACCCGAGAGCTACCCGTAGAGGGCCGGCGGCGGCGGGAGCCGAGTGGGTC      a
           T   T    C        •••T  T  T      G              b
       T       T    C           •••  T      G              d

 529 GGGCGGGGCCGAGCCGGGCCG•GGGCCGTGTGGTGGCCGGGCAGCGGCCG      a
                          T           G        G            b
                          T           G    ••••••A          d

 578 GGCCGGCGGACGGCGGGATGGGCTGCACCGTGAGCGCTGAGGACAAGGCG      a
                                              C             b
                     A                        C             d
                        MetGlyCysThrValSerAlaGluAspLysAla    e

 628 GCGGCCGAGCGCTCCAAAATGATCGACAAGAACCTGCGGGAAGACGGCGA      a
                   T  G                        G     A      b
       A           T  G                        G            d
     AlaAlaGluArgSerLysMetIleAspLysAsnLeuArgGluAspGlyGl     e

 678 GAAGGCGGCGCGGGAGGTGAAGTTGCTGCTCTTGG]                   a
                                  G                         b
             A                  T  G  A                      d
     uLysAlaAlaArgGluValLysLeuLeuLeuLeu                     e
                                          { Intron A
                                          GTGAGGCCGCGTCCT   a
 728 GCGCTGGGACCCCTGATTCCCTACCCAAATTCCCTACTTTGACCTGCGGA      a
 778 CTAGTGTTTCGAACTCCCACACTCGGCCTGGACTTAACCCCGTAGACCCG      a
 828 CCTGGCAAGGACATACAAATAAATAATGGATCAAAACTCAGGGTTGGCCT      a
 878 AGACCTCTCAGATCCAGAGCCCAGACTCTAGATCAGACTCAACTCATCCC      a
 928 AAACCCCGGGCCCCCAAACCCAGACACTGGTGCCTCAACATCCGCTGCCC      a
 978 AGAATT                                                 a
```

FIG. 4. **Sequence of the porcine G protein α_{i-2} subunit gene and its immediate 5′-flanking sequences as compared with other sequences.** The porcine G-protein α_{i-2} subunit gene (*a*) and its deduced amino acid sequence of exon 1 (*e*) were compared to the published sequences of the human α_{i-2} subunit gene (*b*) of Itoh *et al.* (24) and the rat olfactory α_{i-2} subunit cDNA (*d*) of Jones *et al.* (29). Only nucleotides that are different from *sequence a* are depicted. A *bullet* in the sequence (●) indicates a missing base. *Broad inverted arrows* above a sequence (⇓) indicate the minor and major transcription start sites in the porcine α_{i-2} subunit gene. An *upward narrow arrow* (↑) indicates the beginning of the rat olfactory α_{i-2} subunit cDNA. For *line c* only, *asterisk* (*) indicate there is no nucleotide change as compared to *sequence a*. The *underlined sequences* indicate potential regulatory sequences including GC boxes (CCGCCC), CAAT boxes (GCAATC), and TGTGG sequences. Note the human α_{i-2} subunit gene sequence of Weinstein *et al.* (26) differs insignificantly from *sequence b* except for a nucleotide addition at position 169, and four nucleotide substitutions at positions 177 and 187–189 in their genomic sequence.

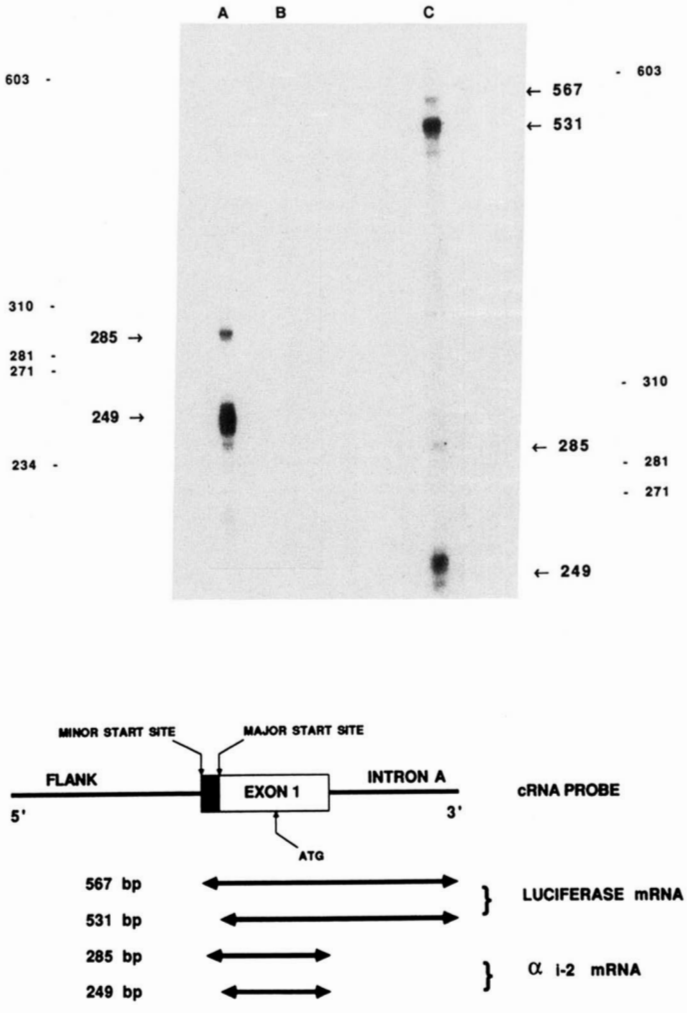

FIG. 5. **S_1 nuclease analysis of porcine LLC-PK_1 renal cell mRNA.** RNA was isolated from LLC-PK_1 cells and LLC-PK_1 cells transfected with the plasmid 10–4 LUC. RNA was quantitated as described under "Experimental Procedures" using a uniformly labeled ^{32}P cRNA probe as seen in the lower illustration. RNA duplexes were subjected to S_1 nuclease digestion. The predicted protected fragments from this reaction are shown as *double-headed arrows* (↔). *Lane A*, LLC-PK_1 mRNA (5 μg); *lane B*, Yeast tRNA (20 μg) contains no protected fragments; *lane C*, LLC-PK_1 total RNA (20 μg) transfected with the 10–4 LUC plasmid. Note: molecular mass markers run with *lanes A* and *B* are shown on the left, whereas the same markers run with *lane C* are shown on the right.

TABLE I

Luciferase activity in LLC-PK_1 cells transfected with chimeric firefly luciferase genes

Plasmid	Length of α_{i-2} 5′ flank	5′ flank direction	No. of transfections	Mean light units ± S.D. normalized to psV2pap
poLUC	0		13	3,422 ± 1,226
10–4 LUC	0.6 kb	+	14	57,560 ± 15,396
		−	15	5,494 ± 2,251
13–2 LUC	11 kb	+	11	118,410 ± 39,967
		−	10	293 ± 110

the transcriptional regulation of the gene in renal epithelium as well as other tissues.

Studies from our laboratory indicated there is a selective alteration of G-protein α subunit levels during differentiation of LLC-PK_1 cells which was suggested by the acquisition of tonic inhibition of adenylyl cyclase by GTP in this renal cell type following polarization. The mRNA for α_s was abundantly expressed in this cell type during differentiation in contrast to the mRNA for α_{i-2}. By comparison, α_{i-2} mRNA is much less abundant and was only detected by day 4 and declined rapidly by day 7 of culture concurrent with the appearance and disappearance of the α_{i-2} subunit at the basolateral membrane.

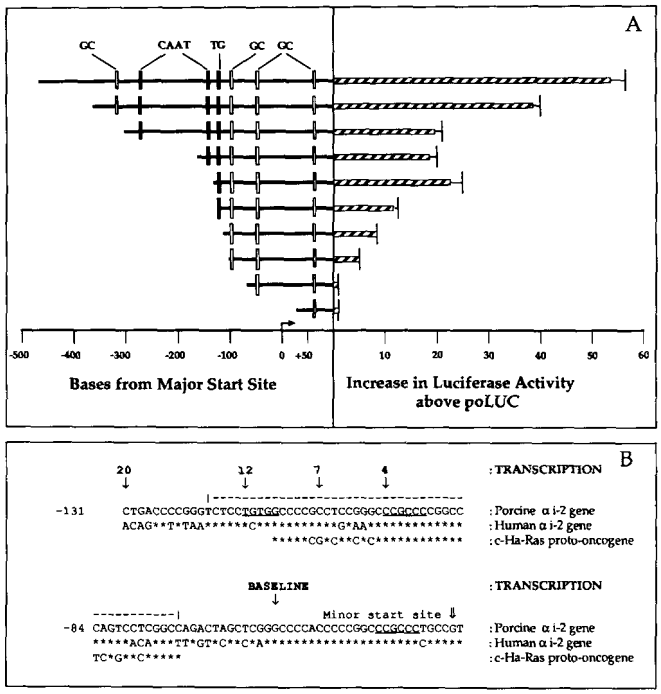

FIG. 6. **Determination of the gene promoter by deletion analysis of the porcine G protein α_{i-2} subunit gene 5′ flank.** *Panel A*, serial deletions of the plasmid (10–4 LUC-(*Xma*III)) at the left of *panel A* were transfected into LLC-PK$_1$ cells and examined for luciferase activity depicted at the right of *panel A*. Luciferase activity results are expressed as fold increases in light units ± S.E. of constructs above poLUC, normalized for human placental alkaline phosphatase activity in each of 19 individual deletion experiments. Baseline light units/heat-insensitive alkaline phosphatase units for poLUC ranged from 300 to 1500 in these experiments with a mean of 1000 as previously reported by Brasier *et al.* (36). *Abbreviations* above the *rectangular boxes* in the *left panel* indicate the potential regulatory sequences: CG (CCGCCC), CAAT (GCAATC), and TG (TGTGG). *Panel B*, determination of the gene promoter by deletion analysis and by comparison to conserved sequences between the human α_{i-2} subunit gene of Itoh *et al.* (25) and the promoter region of the human c-Ha-*ras* proto-oncogene of Ishi *et al.* (45). A *broad inverted arrow* (⇓) indicates the minor transcription start site in the porcine α_{i-2} subunit gene. *Asterisks* (*) indicate there is no nucleotide change as compared with the porcine sequence. The *underlined sequences* indicate potential regulatory sequences including GC boxes (CCGCCC) and TGTGG sequences. *Dashed lines* bordered by *vertical lines* above a sequence (|– – –|) indicate the extent of the porcine α_{i-2} subunit promoter region. A *narrow inverted arrow* (↓) indicates the 5′ nucleotide position of chimeric α_{i-2}-luciferase gene constructs. The *number above the arrow* indicates the fold increases in luciferase activity of these constructs above poLUC that reflect transcription of the gene. *Negative numbers* indicate nucleotide distance from the major transcription start site of the gene.

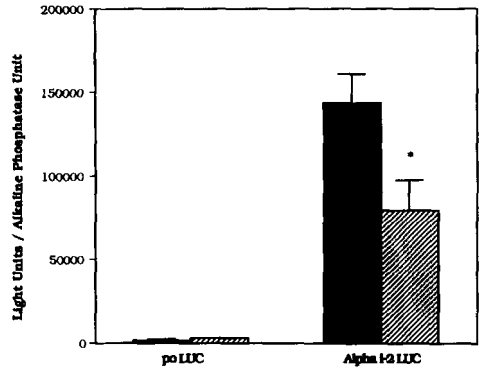

FIG. 7. **Transcription of luciferase plasmids in renal epithelial cells.** poLUC or 13-2 LUC were cotransfected with pRShGRα into LLC-PK$_1$ cells and examined for luciferase activity 16 h after treatment with (*striped bars*) or without (*solid bars*) dexamethasone 10^{-8} M. Luciferase activity results are expressed as mean light units ± S.E., normalized for human placental alkaline phosphatase activity in each of six individual experiment. * $p < 0.01$.

These findings suggested a transcriptional activation of the gene to produce relatively unstable mRNA transcript by day 4 and its deactivation by day 7. These findings are consistent with the variable expression of the gene in other tissues (29). Similar differences in tissue expression are found for another pertussis toxin sensitive α isoform α_o which is highly expressed in neural but not peripheral tissues (46). Its differing content in rat cardiocytes and rat pituitary GH4 cells results from a differential regulation of α_o protein translation in these cells (47).

We have previously shown that transcriptional activation of a chimeric mouse metallothionein I-α_{i-2} gene in these cells produces a 3-fold increased accumulation of α_{i-2} subunits on their basolateral membranes but not their cytosol (24). In other studies the modest increase in an α_{i-2} subunit correctly targeted to its membrane-associated enzyme effector was sufficient to attenuate vasopressin-mediated stimulation of adenylyl cyclase in these cells. Hence, a modulation of transcription and translation of α_{i-2} subunits may act as a control point for the response of renal epithelia to vasopressin.

To understand the transcriptional regulation of the α_{i-2} gene in LLC-PK$_1$ cells, we isolated porcine genomic clones encoding it. The primordial mammalian divergence that produced porcine and primate species occurred approximately 50 million years ago. S$_1$ nuclease mapping of exon 1 revealed its length to be 249 and 285 nucleotides in the porcine gene as compared with 252 and 307 in the human gene. This finding suggested that the 5′-flanking regulatory cis-acting sequences that mediated transcriptional activity were conserved through evolution. The sequence identity between these genes 5′ flanks was 78%. Importantly, the arrangement of putative regulatory elements in the 5′ flanks of these genes such as GC boxes (CCGCCC) CAAT boxes (GTCAATC), and TGTGG-like motifs have been retained (43, 48).

The 5′-flanking region of the porcine α_{i-2} subunit gene shared several characteristics with those of other signal transducing and growth control genes, including the c-Ha-*ras* proto-oncogene (45), the epidermal growth factor receptor gene (49), and other G-protein α subunit genes ($\alpha_{i-1,\ i-3,\ s,\ \text{and}\ t-r}$) (25, 50, 51), that may influence their expression. These characteristics include (i) a high guanosine and cytosine content, (ii) a high frequency of CpG dinucleotides, (iii) multiple GC boxes, (iv) no typical TATAA boxes, (v) absent or atypically placed CAAT box-like sequences, and (vi) multiple transcriptional start sites. The porcine α_{i-2} subunit gene promoter was found in an area that is 67% G-C rich, with 42 CpG *versus* 53 GpC dinucleotides. CpG dinucleotide sequences are potential sites for methylation at the 5-position of cytidine and occur infrequently in genomic DNA except in the regulatory regions of genes (52). In general there is an inverse correlation between the state of methylation of a gene's promoter and its transcriptional activity. This may occur by steric interference with the binding of transcription factors or alteration of chromatin structure (53). Methylation and demethylation events can occur selectively in the same cell resulting in the activation of some genes and the deactivation of others (54). It is notable that following the early differentiation of preadipocytes to adipocytes, there is a differential alteration in the expression of α subunit isoforms (14). Differentiation of these cells is induced by agents which demethylate chromatin (55, 56).

Utilizing a sensitive firefly luciferase reporter gene, we have identified and mapped several cis-acting elements in the 5′-flanking region of the α_{i-2} subunit gene that contributed to its basal transcriptional expression. Analysis of successive 5′-flanking region gene deletions suggested multiple regulatory sequences that are the likely enhancer and promoter elements. The minimal promoter activity was found between positions 333–404. The porcine gene contained two apparent motifs required for basal transcription that were inferred by 1) deletion analysis and 2) sequence comparison to the human c-Ha-*ras* proto-oncogene and the human α_{i-2} subunit genes. These motifs were a GC box at position 368, an AP_2-binding site at position 351, and a TGTGG sequence at position 349. The latter motif which functions as an adenovirus enhancer element (48) may function as a substitute TATAA box sequence which normally positions RNA polymerase II at unique transcription start sites (57). The homologous c-Ha-*ras* proto-oncogene promoter which lacks this sequence has numerous transcription start sites in contrast to the α_{i-2} gene which has only a major and a minor transcription start site (45).

The presence of downstream silencer elements in the α_{i-2} gene was suggested by an increased transcriptional activity following the 3′ deletion in 10–4 LUC of positions 578–983. Decreased luciferase activity of this DNA segment might be attributable to the production of missense translation frames by the use of a different upstream initiator codon. However, the luciferase cDNA has a Kozak consensus sequence preceding its initiator codon that is absent in exon 1 of the porcine α_{i-2} gene. The Kozak consensus sequence preferentially orients the translation apparatus for the use of immediate downstream initiator methionine sequences (58). Furthermore, a similar spanning region for the porcine α_{i-3} gene did not reduce its luciferase activity. Negative modulation of the gene in this area may contribute, in part, to the limited expression of α_{i-2} mRNA in LLC-PK_1 cells in contrast to α_s mRNA.

The existence of three potential areas containing enhancer sequences were also found. The first enhancer located 5′ to position 1 in clone 10–4 was suggested by the increased transcriptional activity of 13–2 LUC (11-kb flank) *versus* 10–4 LUC (0.6-kb flank) (Table I). The second and third enhancers were located between positions 1–111 and 112–172 (Figs. 3*A* and 5) as proven by deletions in these areas of (10–4 LUC-(*Xma*III)). The second enhancer area contains a sequence CGCCTCC at positions 52–58 that is also found at position 357–363 in the minimal promoter area. The third enhancer area contained two overlapping GC boxes at positions 152–162 that contained a conserved sequence GCCCGCCCC which was present at positions 366–374 in the minimal promoter. Notably, although four GC boxes and two CAAT boxes were found as potential regulatory elements, only the two GC boxes at positions 152–157 and 368–373 may contribute to transcriptional activity of the gene as determined by deletion analysis.

Cis-acting sequences such as cyclic AMP response element and glucocorticoid responsive element sites that act as enhancers or silencers for the binding of hormone induced transactivating proteins such as cyclic AMP response element-binding protein (for cyclic AMP) and ligand occupied steroid receptors can function at great distances from the promoter. Only a consensus cyclic AMP response element was found in the immediate 300-bp region of the promoter (42, 43). As our clones encompass at least 11 kb of 5′-flanking sequence, to determine if glucocorticoid responsive element sites exist, we attempted to functionally identify them in luciferase reporter gene assays. Transcription of the α_{i-2} gene was inhibited in dexamethasone-treated renal cells complemented with the human glucocorticoid receptor. These findings are consistent with previous observations that corticosterone inhibits α_{i-2} mRNA and protein expression in rat cerebral cortex (18).

Having isolated genomic clones that encode 5′-flanking regions of the porcine α_{i-2} subunit gene and characterized several potential cis-acting elements that contribute to its transcriptional activity, it is now possible to begin to identify the protein factors that act in "trans" to modulate its activity. These studies provide a basis for elucidating cis-acting DNA sequences and trans-acting protein factors that act in concert to control the transcriptional regulation of the α_{i-2} gene during renal epithelial cell differentiation. Furthermore, the influence on α_{i-2} subunit gene transcription of hormones, second messengers, and lymphokines known to alter α_{i-2} expression in other tissues can now be examined in renal epithelia.

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