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Tissue-Specific Expression and Regulation by 1,25(OH)$_2$D$_3$ of Chick Protein Kinase Inhibitor (PKI) mRNA

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The heat-stable protein kinase inhibitor (PKI) protein is a specific and potent competitive inhibitor of the catalytic subunit of cAMP-dependent protein kinase (PKA). Previously, it has been shown that vitamin D status affects chick kidney PKI activity: a 5- to 10-fold increase in PKI activity was observed in kidneys of chronically vitamin D-deficient chicks and treatment with 1,25-dihydroxyvitamin D$_3$ (1,25[OH]$_2$D$_3$) in cultured kidney cells resulted in a 95% decrease in PKI activity. The authors have recently cloned the cDNA for chick kidney PKI and have used the coding sequence to study the regulation of PKI mRNA. Northern analysis showed the expression of two PKI messages, which are 2.7 and 3.3 kb in size. These mRNAs are expressed in brain, muscle, testis, and kidney, but not in pancreas, liver, or intestine. PKI mRNA steady-state levels are downregulated by 47% in kidneys from vitamin D-replete chicks as compared to vitamin D-deficient chicks. PKI mRNA levels in brain, muscle, and testis are not affected by vitamin D status. Treatment of primary chick kidney cultures treated with $10^{-7}M$ 1,25(OH)$_2$D$_3$ for 24 h resulted in a 20–30% decrease in PKI mRNA. 1,25(OH)$_2$D$_3$ treatment does not affect the stability of PKI mRNA as determined by treatment of cell cultures with actinomycin D. This study shows that 1,25(OH)$_2$D$_3$ directly and tissue-specifically downregulates PKI mRNA in the chick kidney.

Key Words: Protein kinase inhibitor (PKI); cAMP-dependent protein kinase (PKA); vitamin D; 1,25-dihydroxyvitamin D3 (1,25[OH]$_2$D$_3$).

Introduction

cAMP-dependent protein kinase (PKA), which is activated by increases in intracellular cAMP levels, mediates a variety of cellular processes in eukaryotic cells such as hormone responsiveness, cell growth and division, and gene expression. In the cytosol, cAMP binds to the regulatory subunit of inactive PKA resulting in dissociation of the PKA tetramer into a regulatory subunit dimer and active cAMP-independent catalytic subunits. In addition to cAMP, a potentially important intracellular regulator of cAMP-dependent PKA is the endogenous protein kinase inhibitor (PKI) *(1,2)*. PKI has the potential for inhibiting intracellular cAMP-dependent protein phosphorylation and, recently, it has also been shown to have an effect on the nuclear localization of the PKA catalytic subunit and its attendant effects on gene expression *(3,4)*.

The 11 kDa PKI is a heat- and acid-stable protein found in many vertebrate tissues *(5–8)* that is a potent competitive inhibitor of the PKA catalytic subunit with K_i values in the 5–11 nanomolar range *(6,9)*. PKI has no known enzymatic activity, nor does it bind cAMP or serve as a substrate for the kinase *(2,6)*.

PKI activity has been shown to be under hormonal and nutritional control *(8,10–15)* and it has been suggested that the protein may play an important role in regulating cAMP-dependent PKA activity under physiological conditions. Several years ago, we observed that vitamin D-deficient chickens have reduced kidney PKA activity and markedly increased PKI activity in comparison to vitamin D-replete birds *(14)*. These differences in both PKA and PKI activity were specific for the kidney with no effect of vitamin D status in brain, heart, skeletal muscle, or pancreas *(8)*. More recently, the authors have shown that 1,25-dihydroxyvitamin D$_3$ (1,25[OH]$_2$D$_3$), the active steroid hormonal form of vitamin D, directly decreases PKI activity in primary cultures of chick kidney cells *(15)* in a dose- and time-dependent manner.

It has been hypothesized that this upregulation of PKI by vitamin D deficiency could contribute, along with homologous desensitization *(16)*, to the classical "blunting" the renal response to parathyroid hormone (PTH) associated with vitamin D status *(17)*. Before such a role for PKI in the kidney can be established, an understanding of the molecular mechanisms of PKI regulation in the chick kidney must be obtained. The present study shows that steady state levels

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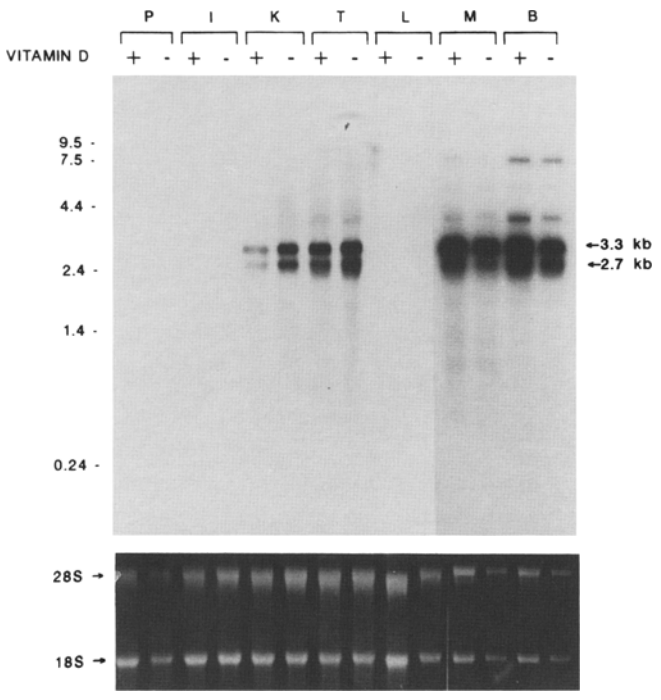

Fig. 1. Northern blot of PKI mRNA in tissues from 3-wk-old vitamin D-deficient or -replete male Leghorn chicks. RNA, 10 μg/lane, was electrophoresed on a 1.2% formaldehyde/agarose denaturing gel (upper portion). Quantity and quality of RNA was visualized by ethidium bromide staining (lower portion). In the upper panel, the samples were transferred and covalently fixed to a nylon membrane and hybridized with a ^{32}P-labeled PKI antisense DNA probe. Indicated tissues are as follows: P, pancreas; I, intestine; K, kidney; T, testis; L, liver; M, muscle; B, brain. Vitamin D status is denoted by + for replete birds and – for deficient birds (*see* Materials and Methods). RNA size markers (BRL, Gaithersburg, MD) are indicated to the left and sizes of bands corresponding to PKI messages are indicated to the right. The lower panel shows ethidium bromide staining of gel prior to transfer.

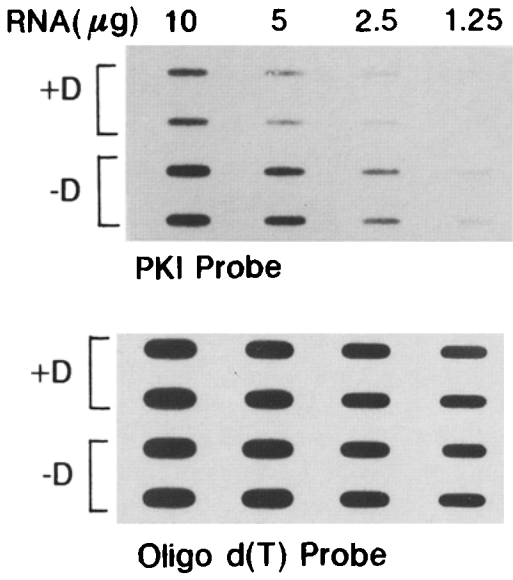

Fig. 2. Representative slot blot of kidney total RNA from +/– D chicks. Serial dilutions of total RNA isolated from +/– D chick tissues were denatured prior to blotting onto duplicate nylon membranes, covalently fixed, and probed with either chick kidney PKI (upper panel) or oligo d(T) (rawer panel), for normalization of RNA loadings. The amount of RNA in each sample is indicated above each column. Rows containing + or – D samples are indicated between panels.

of PKI mRNA are regulated tissue specifically by vitamin D status in chick kidney and by $1,25(OH)_2D_3$ in primary cultures of chick kidney cells.

Results

Expression of PKI mRNA and Vitamin D-Dependent Downregulation in Various Chick Tissues

Our chick kidney cDNA clone encodes a 76 amino acid protein that is more similar in sequence and length to the mammalian PKIα isoform than mammalian PKIβ *(18)*. Using the coding region of the chick kidney PKI cDNA as our probe, Northern analysis of total RNA isolated from tissues of 3-wk-old vitamin D-deficient and -replete chicks was performed to look at PKI tissue distribution. Levels of PKI mRNA expression are highest in chick brain and skeletal muscle with decreasing levels of expression in chick testis and kidney, respectively (Fig. 1). Expression of PKI mRNA was not detected with the kidney probe in chick intestine, liver, or pancreas, even after long exposures of the X-ray film. Two major bands corresponding to mRNA of 2.7 and 3.3 kb in size are observed in all four tissues expressing PKI. Additionally, the chick kidney PKI probe detects two minor bands in muscle and brain that correspond to 4.0 and 7.5 kb mRNAs. These may represent incompletely processed heterogeneous nuclear RNA. The size of chick PKI mRNAs and its tissue distribution gives further evidence of our chick PKI's similarity to mammalian PKIα.

Northern analysis of chick tissues showed a significant downregulation of PKI mRNA steady-state levels in the kidney of vitamin D-replete chicks. Both the 2.7 and 3.3 kb messages of PKI were downregulated by vitamin D (Fig. 1). An apparent vitamin D upregulation of PKI mRNA levels in chick skeletal muscle and brain (Fig 1, lanes 11–14) is a result of unequal loading of total RNA levels as assessed by ethidium bromide stained samples (Fig. 1, lower panel). This observation was not made in other experiments.

To determine more quantitatively whether regulation of PKI mRNA by vitamin D status occurred in chick tissues other than kidney, slot blot analysis of mRNA from several chick tissues was performed. Blots of each tissue (kidney, testis, muscle, and brain) were prepared from three independent of vitamin D-deficient and -replete chicks and a typical blot is shown (Fig. 2). A summary of all the accumulated data (Fig. 3) shows that the –D/+D ratio of kidney PKI mRNA steady-state levels is 1.9 ± 0.2, representing a 47% downregulation of PKI mRNA in the kidney of vitamin D-replete chicks. In contrast, the –D/+D ratio of PKI

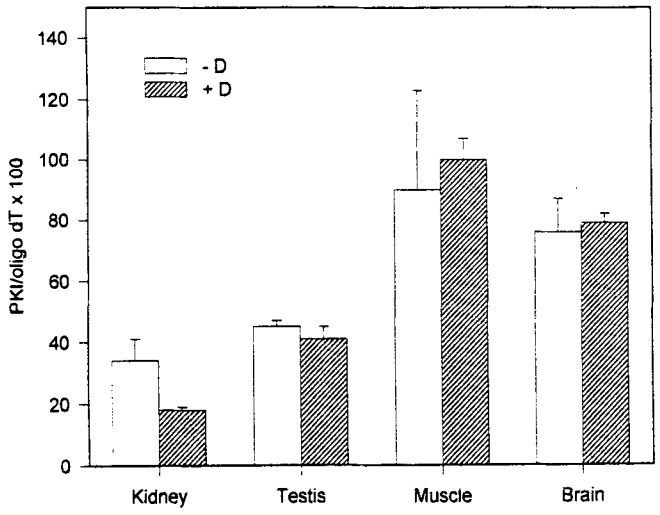

Fig. 3. Individual duplicate slot blots were prepared from three independent preparations of chick kidney, testis, muscle, and brain total RNA. Autoradiograph signals from blots, probed with either ^{32}P-labeled-PKI or -oligo d(T), were quantified by an LKB Ultroscan XL laser densitometer. The signal intensities from hybridization with the PKI probe were normalized to corresponding intensities from the oligo d(T) probe. The results in the graph are presented as a ratio of PKI/oligo d(T), in vitamin D-deficient, (–D), and -replete, (+D), chick tissues. Data (in arbitrary units ×100) are expressed as mean ± SD (n = three independent experiments). Vitamin D significantly decreased PKI mRNA in chick kidney ($p < 0.05$, by paired t test).

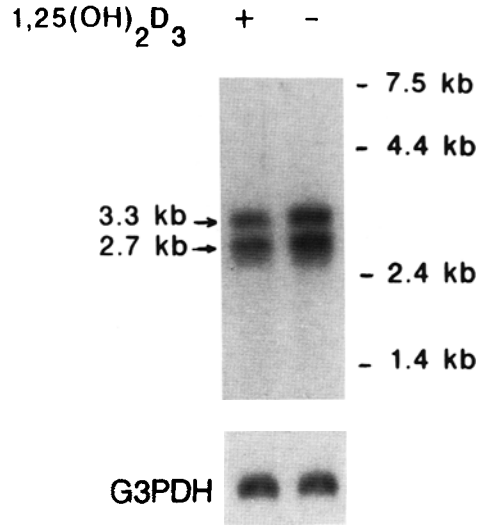

Fig. 4. Representative northern blot of PKI mRNA in cultured chick kidney cells. Twenty-four hours prior to harvest, cells were changed to serum-free medium. At this time, and again at 12 h prior to harvest, cells were treated with either $10^{-7}M$ $1,25(OH)_2D_3$ or vehicle (ethanol). Total RNA was isolated and 10 µg of RNA electrophoresed on a 1.2% denaturing gel, transferred to a nylon membrane, and covalently fixed. Blots were hybridized with an antisense ^{32}P-labeled PKI probe, followed by stringent washes. The northern blot shown is typical of the results of several cell culture experiments. In the upper panel, treatment with $1,25(OH)_2D_3$ is indicated above the blot. Bands corresponding to the PKI 2.7 and 3.3 messages are indicated by arrows to the left. The lower panel shows the same blot probed, after stripping, with G3PDH for comparison of loading. G3PDH mRNA levels are not affected by vitamin D status *(31)*.

mRNA levels was not significantly different from 1.0 in testis, muscle, or brain.

Downregulation of PKI mRNA by $1,25(OH)_2D_3$ in Primary Cultures of Chick Kidney Cells

The above results demonstrate that the previously observed kidney-specific decrease in PKI activity brought about by vitamin D repletion *(8)* involves downregulation of steady-state mRNA levels. The authors next wanted to determine whether the $1,25(OH)_2D_3$-mediated decrease in PKI activity in cultured chick kidney cells *(15)* was also because of downregulation of PKI mRNA levels. Northern analysis of RNA isolated from primary cultures of chick kidney cells treated with $10^{-7}M$ $1,25(OH)_2D_3$ or vehicle for 24 h was performed. Consistent with the results from fresh tissue (Fig. 1), PKI mRNA is downregulated when cells are treated with $1,25(OH)_2D_3$ (Fig. 4). Slot blot analysis showed a 30% downregulation of PKI mRNA levels as a result of 24 h treatment with $1,25(OH)_2D_3$ in cell cultures (data not shown). The results of these experiments demonstrate that the effect of vitamin D status on kidney PKI mRNA can be replicated by $1,25(OH)_2D_3$ in cultured kidney cells, indicating a direct effect of the hormone on PKI mRNA levels.

To determine whether the $1,25(OH)_2D_3$-dependent decrease in cell culture PKI mRNA levels was caused by an alteration in stability of the RNA message, $1,25(OH)_2D_3$- and vehicle-treated cell cultures were incubated with 5 µg/mL actinomycin D, an inhibitor of RNA synthesis. From the northern blot in Fig. 5A, two qualitative observations can be made. First, a decrease in PKI mRNA steady-state levels is observed when cells are treated with $10^{-7}M$ $1,25(OH)_2D_3$ (4 right lanes) as compared to that found in vehicle-treated cells (4 left lanes), which is in agreement with the results reported in Fig. 3. Second, PKI mRNA levels from both vehicle- and $1,25(OH)_2D_3$-treated samples decrease over the 8-h incubation with actinomycin D when compared to their respective 0 time incubations. For reasons that are not clear, in cultured kidney cells the lower molecular weight PKI mRNA tends to predominate, whereas in fresh kidney tissue, the two mRNA species are approximately equally abundant.

Slot blot analysis was performed on these RNA samples to determine more quantitatively whether $1,25(OH)_2D_3$ treatment had an effect on PKI mRNA stability. Oligo d(T) was used to normalize RNA sample loadings. Normalized PKI mRNA levels in $1,25(OH)_2D_3$-treated cells are presented as the percent of the normalized values in vehicle-treated cells (Fig. 5B). The fact that this percentage is unaffected by $1,25(OH)_2D_3$-treatment indicates that the $1,25(OH)_2D_3$-mediated downregulation of PKI mRNA does not occur through alteration in message stability, but may be occurring through effects on gene transcription.

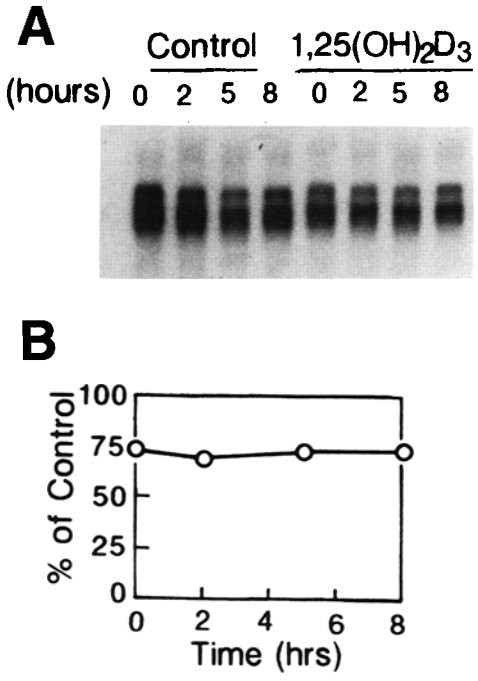

Fig. 5. Northern blot of PKI mRNA from cultured chick kidney cells in the presence of actinomycin D **(A)** and density of PKI mRNA signal normalized to oligo d(T) **(B)**. Cultured kidney cells were changed to serum-free medium 24 h prior to harvest and treated with $10^{-7}M$ $1,25(OH)_2D_3$ or vehicle (ethanol) in two 12-h doses. Actinomycin D (5 μg/mL) was then added to cell culture, and total RNA harvested at the times indicated above the blot. PKI mRNA levels were quantified by slot blot analysis and densitometric scans of slot blots of PKI mRNA levels were normalized to oligo d(T). Normalized PKI mRNA levels in 1,25(OH)-treated cells are presented as the percent of the normalized values in vehicle-treated control cells.

Thus, the $1,25(OH)_2D_3$-mediated downregulation of PKI mRNA does not occur through alteration in message stability, but may be occurring through effects on gene transcription.

Discussion

This is the first study showing that PKI is a regulatable component of the cAMP-dependent signaling system at the level of its mRNA by a steroid hormone and that this regulation occurs in a tissue-specific manner. The authors now show that PKI mRNA steady-state levels are downregulated specifically in the kidney by the seco-steroid hormone, $1,25(OH)_2D_3$. The evidence presented here confirms our earlier observation that PKI activity is downregulated by $1,25(OH)_2D_3$ in the chick kidney *(15)*. Earlier studies have shown that PKI activity is regulated in a variety of experimental systems. These include induction of PKI levels in heart following starvation and refeeding of rats *(10)*, and repression in rat adipose tissue and pancreas after experimentally induced diabetes *(11)*. Stimulation of proliferation of Chinese hamster ovary cells by serum increased PKI levels *(12)*, as did treatment of Sertoli cells in culture with follicle-stimulating hormone (FSH) *(13)*. Recently, PKI mRNA was shown to be regulated in a developmental-specific manner *(19)*.

In addition to inhibiting the phosphorylation activity of PKA, PKI has been shown to regulate the localization of the catalytic subunit of PKA *(3,4)*. It has been shown that PKI contains a nuclear export signal (NES) of approx 15 amino acids, which is distinct from the kinase inhibition domain. This NES leads to the accumulation of PKA catalytic subunit within the cytoplasm *(4)*. Although the physiological consequences of PKI downregulation by $1,25(OH)_2D_3$ in the kidney have yet to be established, it is possible that a decrease in PKI activity may play a role in alteration of PKA-sensitive gene expression.

The seco-steroid hormone, $1,25(OH)_2D_3$, binds to its nuclear receptor to affect the transcription of many genes (for a review, *see* ref. *20*). The vitamin D receptor is a member of the steroid-thyroid-retinoid receptor gene family of nuclear transcription factors. In most studies involving regulation of vitamin D sensitive genes, the transcriptional effect is positive. Much less is known about negative response elements. Examples of negative regulation of gene expression by $1,25(OH)_2D_3$ are found in the genes for parathyroid hormone (PTH) *(21)*, calcitonin *(22)*, α1(I) collagen *(23)*, and c-*myc* *(24)*. The findings show that $1,25(OH)_2D_3$ alters PKI mRNA steady-state levels in chick kidney; thus PKI joins the expanding group of genes that are negatively regulated by $1,25(OH)_2D_3$. The vitamin D-dependent downregulation of PKI mRNA may in part involve binding of the vitamin D receptor to a negative VDRE associated with this gene. Additionally, the factor(s) involved in the regulation would be kidney-specific.

In summary, chick PKI mRNA has two major forms that are 2.7 and 3.3 kb in size and are expressed in high levels in brain and muscle, and to a lesser degree in testis and kidney. The vitamin D-dependent downregulation of PKI mRNA is specific to the kidney and is a direct effect of the active form of the hormone, $1,25(OH)_2D_3$. Experiments are now under way to identify vitamin D responsive elements or other recognizable elements within the PKI gene promoter that can establish the specific mechanisms of $1,25(OH)_2D_3$ downregulation of chick kidney PKI mRNA.

Materials and Methods

Materials

*Eco*RI and Klenow DNA polymerase I were purchased from Promega (Madison, WI) and thermostable AmpliTaq DNA polymerase from Perkin-Elmer (Norwalk, CT). Radiolabeled nucleotides were purchased from Amersham (Arlington Heights, IL). Vitamin D_3 was obtained from Philips-Duphar (Amsterdam, The Netherlands). $1,25(OH)_2D_3$ was generously provided by Dr. M. Uskokovic of Hoffman-LaRoche (Nutley, NJ). Oligonucleotide primers were synthesized at the Biotechnology Instrumentation Facility,

University of California (Riverside, CA). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was provided by Dr. A. Dugaiczyk, University of California.

Animals

White Leghorn cockerels (Hyline Int., Lakeview, CA) were obtained on the day of hatching and raised in the absence of sunlight on a 0.6% calcium and 0.4% phosphorous rachitogenic diet *(25)* with the addition of methionine (250 g/500 kg diet). Chicks were given oral doses of 13 nmol vitamin D_3 or vehicle (ethanol-1,2-propanediol [1:1, v/v]) three times a week. This vitamin D_3 dose was based on previous experiments in which maximum downregulation of PKI activity was observed. Vitamin D status was confirmed by body weight and serum calcium levels in three independent experiments. Average body weights ranged from 150–175 g for vitamin D replete chicks and 85–110 g for vitamin D deficient chicks. The mean ratio of +D/–D serum calcium levels was 1.6.

PCR Amplification of Coding Sequence

Primers N21-PKI, (5'-ATGACTGATGTGGAATCTACA-3') and C21-PKI, (5'-GCTTTCTTGCTTTGCTGCCTC-3') were used to amplify the coding region of chick kidney PKI from 100 ng of cDNA template in a 50 µL reaction volume containing 200 µ*M* of each dNTP, 10 pmol each primer, 2.5 m*M* $MgCl_2$, 10 m*M* Tris, pH 8.4, and 5 U of AmpliTaq DNA Polymerase (Perkin-Elmer, Norwalk, CT). Twenty-five cycles of denaturation (94°C for 60 s), annealing (55°C for 30 s), and extension (72°C for 30 s) were performed. The 228 bp product was subcloned directly into the pCRII plasmid (Invitrogen, San Diego, CA) and transformed into competent INVα' *E. coli* cells according to the manufacturer's protocol. A positive PKI subclone was designated as pCRIIPKI.53 and confirmed by double-stranded plasmid DNA dideoxy sequencing *(26)*.

Total RNA Isolation, Northern Blot Analysis, and Slot Blot Analysis

Kidney, brain, muscle, intestine, pancreas, testis, and liver tissue were removed from 3-wk-old vitamin D-replete or vitamin D-deficient chicks and snap-frozen in liquid nitrogen. In three independent experiments, the number of birds per treatment group ranged from 6 to 25. Tissue was pulverized prior to homogenization and total cellular RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform procedure described by Chomczynski and Sacchi *(27)* with modifications as described by Puissant and Houdebine *(28)*. Ten micrograms of total RNA was electrophoresed on a 1.2% agarose–formaldehyde Northern gel *(29)*, visualized by ethidium bromide staining for quantity and quality of RNA, and photographed. RNA was transferred to a Nytran MaxiStrength nylon membrane (Schleicher & Schuell, Keene, NH), dried, and covalently fixed to the membrane by W crosslinking. Ethidium bromide was used for this purpose because the lack of an effect of vitamin D status on G3PDH mRNA levels had not yet been confirmed for all the tissues under investigation. Slot blot samples of the indicated tissues were prepared from 1:1 serial dilutions of total RNA, denatured in formaldehyde/SSC at 60°C, and blotted under low vacuum using an ABN VacuSlot manifold (Emeryville, CA). Dilutions were applied to a Nytran MaxiStrength nylon membrane in quadruplicate and separate membranes were prepared for each probe (PKI and oligo d[T]) using the same dilutions. Slot blots were dried and RNA covalently fixed to the membrane by UV crosslinking. Hybridizations were performed with Rapid Hybridization solution (Amersham) according to manufacturer's protocol. A single-stranded ^{32}P-labeled DNA probe, complementary to PKI mRNA, was synthesized in a specific primed Klenow reaction using the PKI C21-PKI primer. Purified probe was added at a final concentration of 10^6 cpm/mL and blots were hybridized for 2 h at 65°C, washed under high stringency conditions, and exposed to X-ray film. Slot blot autoradiographs were scanned with an LKB laser densitometer and intensities of the quadruplicate signals were averaged. The average intensity of each dilution was normalized with the average intensity of the corresponding dilution after hybridization with oligo d(T). Oligo $d(T)_{12-18}$ was 5' end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (USB, Cleveland, OH) according to manufacturer's instructions. Slot blots probed with oligo d(T) were hybridized in 6X SSPE/0.1% SDS at room temperature overnight followed by stringent washes and exposed to X-ray film.

Cell Culture, Total RNA Isolation, and Northern Blot Analysis

Primary cultures of chick kidney cells were prepared from 3-wk-old vitamin D-deficient chicks as described previously *(30)*. Cells were cultured in Minimal Essential Medium supplemented with 5% fetal calf serum and harvested on the fourth day after plating. Twenty-four hours prior to harvesting, cultures were changed to serum-free medium containing 5 µg/mL insulin, at which time treatment with $1 \times 10^{-7}M$ $1,25(OH)_2D_3$ or vehicle (ethanol) was begun. Total RNA isolation and northern blot analysis was performed as described for fresh tissue.

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