Regulation of the Na-K-ATPase β1-subunit promoter by multiple prostaglandin-responsive elements

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Matlhagela, Keikantse, and Mary Taub. Regulation of the Na-K-ATPase β1-subunit promoter by multiple prostaglandin-responsive elements. Am J Physiol Renal Physiol 291: F635–F646, 2006. First published February 14, 2006; doi:10.1152/ajprenal.00452.2005.—Renal prostaglandins modulate the activity of a number of the transport systems in the kidney, including the Na-K-ATPase. Not only do prostaglandins have acute affects on renal Na-K-ATPase, but in addition prostaglandins have chronic affects, which include regulation at the transcriptional level. Previously, we have presented evidence that one such prostaglandin, PGE1, stimulates the transcription of the human Na-K-ATPase β1-subunit gene in Madin-Darby canine kidney cells via cAMP- and Ca2+-mediated pathways (Taub M, Borsick M, Geisel J, Matlhagela K, Rajkhowa T, and Allen C. Exp Cell Res 299: 1–14, 2004; Matlhagela K, Borsick M, Rajkhowa T, and Taub M. J Biol Chem 280: 334–346, 2005). Evidence was presented indicating that PGE1 stimulation was mediated through the binding of cAMP-regulatory element binding protein (CREB) to a prostaglandin-responsive element (PGRE) as well as Sp1 binding to an adjacent Sp1 site. In this report, we present evidence from EMSAs and DNA affinity precipitation studies that another PGRE present in the Na-K-ATPase β1-subunit promoter similarly binds CREB and Sp1. The evidence that indicates a requirement for CREB as well as Sp1 for gene activation through both PGREs (PGRE1 and PGRE3) includes studies with a dominant negative CREB (KCREB), Drosophila SL2 cells, and PGRE mutants. The results of these studies indicate a synergetic effect between Sp1 and CREB in mediating regulation by PGRE3; while regulation occurring through PGRE1 also involves Sp1 and CREB, the mechanism appears to be distinct.

Madin-Darby canine kidney cells; transport; gene regulation; kidney; eicosanoids

RENAL PROSTAGLANDINS ARE PRODUCTS of arachidonic acid (AA) metabolism by cyclooxygenase, which act as modulators of a number of renal functions, including Na+ transport (29). Investigations concerning prostaglandins’ effects on Na+ transport in tubule epithelial cells in the intact kidney have been complicated by the varying responses of different types of tubule epithelial cells to prostaglandins, as well as by the distinct modes of regulation of prostaglandins by cyclooxygenase 1 (COX-1), as well as by COX-2, which are present in distinct locales in the kidney (20). In addition to responding to exogenous prostaglandins, tubule epithelial cells produce prostaglandins, which act in an autocrine manner (29). This is exemplified in the case of principal cells in the collecting duct, in which endogenous AA is released following the activation of phospholipase A2. The released AA may then either act directly to inhibit apical Na+ influx by the epithelial Na+ channel (ENaC), or the AA may be metabolized to PGE2, so as to maximize basolateral Na+ efflux (45). Of particular interest to this report are the mechanisms by which prostaglandins affect one such basolateral membrane transport system, the Na-K-ATPase.

The Na-K-ATPase is an integral membrane protein that plays an essential role in the physiology of animal cells. The Na-K-ATPase maintains an electrochemical gradient by transporting three intracellular Na+ molecules out of the cells in exchange for three extracellular K+ molecules, in an ATP-dependent manner (18). The electrochemical gradient established by the Na-K-ATPase is required for a number of cellular processes, including cell volume regulation, maintenance of the action potential in excitable cells, and the activity of a number of membrane transport systems (19). In the kidney, the Na-K-ATPase is localized in the basolateral membrane and is integral to the process of sodium reabsorption (12, 13, 18, 24, 27). The electrochemical gradient established by the renal Na-K-ATPase acts as the driving force for the translocation of glucose, phosphate, and amino acids across the apical membrane by Na+/solute cotransport systems (23). Subsequently, these solutes are transported out of the cells’ basolateral membrane by means of another set of transport systems.

The Na-K-ATPase is composed of an α-subunit (110 kDa), responsible for the transport activity, as well as a glycosylated β-subunit (60 kDa). The β-subunit facilitates the correct assembly and transport of the α-subunit into the basolateral membrane of epithelial cells. This process is dependent on α/β heterodimer formation, which is limited by the levels of newly synthesized α- and β-subunits (4, 15). The level of the newly synthesized β-subunit has been found in some cases to be a limiting factor in α/β heterodimer formation (15). In those cases in which the newly synthesized β-subunit is limiting, regulatory changes that affect β-subunit levels ultimately affect overall Na-K-ATPase levels and sodium reabsorption by the kidney.

The activity of the Na-K-ATPase changes in response to changes in the extracellular environment. Acute regulation of the enzyme, which occurs within minutes to hours of a stimulus, generally occurs posttranslationally. Chronic regulation, which occurs within hours to days, involves changes in the numbers of Na-K-ATPases. Included among the regulatory changes that affect the numbers of Na-K-ATPases are changes in the general hormonal milieu as well as in more localized, organ-specific signals (16, 24). In the kidney, hormones that regulate the level of expression of the Na-K-ATPase α- and β-subunits include angiotensin II (30) and vasopressin (31). An understanding of the mechanisms responsible for the regulation of Na-K-ATPase expression is important for elucidating the physiological responses of the kidney to changes in circulating hormones and the effects of the kidney on the general hormonal milieu.

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β-subunit genes (and ultimately modulate Na-K-ATPase levels) include glucocorticoids, mineralocorticoids, and thyroid hormone (5, 10, 14). In addition, endogenously produced effector molecules that play a role in regulating the renal Na-K-ATPase include dopamine, angiotensin, and prostaglandins, the products of AA metabolism by COX (25, 37).

Transcriptional regulation of the Na-K-ATPase may occur via a number of mechanisms. Hormones such as glucocorticoids, mineralocorticoids, and thyroid hormone bind to specific cytoplasmic receptors, which then enter the nucleus as a hormone-receptor complex, and then the hormone-receptor complex binds to regulatory elements located in the promoter region of the target genes (5, 14). Ultimately, changes observed at the transcriptional level affect the level of the Na-K-ATPase in the plasma membrane, which are in addition to any post-transcriptional effects, which may occur as a consequence of hormone treatment. In contrast, effector molecules that act via G protein-coupled receptors, such as prostaglandins, activate signaling pathways including cAMP, PKC, and/or other Ca2+ signaling pathways, which ultimately also affect transcription (8).

Previously, we reported that PGE1 stimulates the activity of the Na-K-ATPase in Madin-Darby canine kidney (MDCK) cells (37, 40) and that the increase in Na-K-ATPase activity caused by PGE1 can be explained by regulation at the transcriptional level (37). The regulation of β-subunit transcription by prostaglandins was examined in detail, using a human Na-K-ATPase β1 promoter/luciferase construct, pHβ1–1141Luc (Fig. 1A) (14, 25, 37). We defined a region within the Na-K-ATPase β1-subunit promoter (~83 to ~182) that is required to elicit the effects of PGE1, as well as a prostaglandin-responsive element (PGRE) within this region (AGTCCCTGC; ~92 to ~100) that was required to elicit a PGE1 stimulation (25). EMSAs indicated that both the cAMP-regulatory element binding protein (CREB) and Sp1 are involved in mediating a PGE1 stimulation by binding to the PGRE and an adjacent Sp1 site, respectively (25). The involvement of the PGRE and adjacent Sp1 sites was also indicated by studies with mutant constructs (25).

While these previous studies indicated the presence of a PGRE with the sequence AGTCCCTGC within the human β1-subunit promoter (~92 to ~100), the 5′ deletion analysis did not necessarily exclude the presence of additional PGREs within the β1-subunit promoter. In this study, we examine the possibility that there are indeed additional PGRE sites. Evidence is presented indicating that indeed a second PGRE is present within the human β1-subunit promoter and that regulation through this PGRE is via a similar, albeit distinct mechanism from that occurring by the previously identified PGRE (at ~92 to ~100).

MATERIALS AND METHODS

Materials

Hormones, human transferrin, PGE1, and other chemicals were from Sigma (St. Louis, MO). Synthetic double-stranded oligonucleotides, medium, fetal bovine serum, soybean trypsin inhibitor, lipofectamine, and Cellfectin Reagent were from Invitrogen (Carlsbad, CA). BioMax MS-2 film, [γ-32P]dCTP and [α-32P]dATP were from PerkinElmer Life Sciences. The Galacto-Star system was from Applied Biosystems (Bedford, MA). The pSVβ-gal plasmid, reporter lysis buffer, as well as the consensus Sp1 and CRE oligonucleotides were from Promega (Madison, WI). The Prism 4 program was obtained from GraphPad Software (San Diego, CA). Nitrocellulose membranes, the Immuno-Star AP Detection Kit, and other reagents for electrophoresis were from Bio-Rad (Hercules, CA). Streptavidin-agarose was from Pharmingen (San Diego, CA), and Drosophila SL2 cells were from the American Type Culture Collection (Manassas, VA).

The affinity-purified rabbit polyclonal antibodies, which were employed in these studies, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-human CREB-1 antibody (C-21, sc-186) recognizes dog CREB, which has an identical sequence to human CREB (NCBI database). Anti-CREB-1 also recognizes ATF-1 and CREM-1, although these proteins have molecular masses below 40 kDa. The epitope recognized by the anti-human Sp1 antibody (H-225, sc-1402X) includes the NH2-terminal amino acids 121–345. The anti-human CREB antibody (A-22, sc-369) is against an NH2-terminus epitope (95% homology between dog and human), whereas the anti-human CREB antibody (A-22, sc-369) is against an NH2-terminal epitope (95% homology between dog and human). The anti-TFIID antibody (N-12, sc-204) is against an epitope mapping at the NH2 terminus of TFIID binding protein (TBP, 96% homology between dog and human). TBP is a component of human TFIID.

Expression Vectors

The Re/RSV-KCREB vector, containing a dominant negative CREB (KCREB), and the empty vector Re/RSV were obtained from Dr. Richard Goodman (Univ. of Oregon) (41). The Drosophila expression vectors pPacO, pPacSp1, and pPacSp1gal were obtained from Dr. Robert Tjian (UC Berkeley) (9). The human Na-K-ATPase β1 promoter/luciferase construct pHβ1–1141Luc (Fig. 1A) was obtained from Dr. Jerry Lingrel (Univ. of Cincinnati) (14). The deletion mutant pHβ1–1141LucΔ71–298, pHβ1–1141Luc was obtained by the digestion of pHβ1–1141Luc with BamHI and Bgl II and ligation using T4 DNA ligase.

The vector pLuc-MCS (Stratagene, La Jolla, CA) contained a minimal promoter with a TATA box linked to the luciferase gene. The cis-reporting plasmid pCRE-Luc (Stratagene) contains four consensus CRE elements (AGGCGACGTCAGAG) in tandem, immediately upstream of the TATA box in pLuc-MCS. Other constructs were created by ligating synthetic oligos into the HindIII/XhoI site immediately upstream of the TATA box. For pLuc-MCS-β72–167, the synthetic oligo (homologous to −167 to −72 of the β1 promoter) (Fig. 1A) was CAGCGATCCA AGCGGGCCCCCT CTATGGCGGG CGGCTCTCTTT GTGCCGGGCC CGGAACCGCG CTCTCGGGCC GAGTCCCTGC CCCCTGGCGCC GGCGATTTGCG. For pLuc-MCS-β72–167 mut PGRE, the insert was homologous to −167 to −72 within the β1 promoter but had mutations in PGRE3, CAGCG ATCCAAAGCG CCCCTCTAGCG CCCGGGCCGCT CTCTTGTGCG GGCGCCGAC CGCGCTCTCG GCCGGCCGATTA GTAAAC- CCTG GCAGCGCCGCA TTGGCG. For pLuc-MCS-β72–167 mut GC Bx 1, the insert was homologous to −167 to −72 within the β1 promoter, with mutations in the GC box (~117 to ~117), CAGCG ATCCAAAGCG CCCCTCTAGCG GCAGCGCCGCT CTCTTGTGCG GGCGCCGAC ATTAACCGTTT CCCCTGGCGCC AGCGCATTTT GCAGCGCCGCA TTGGCG. For pLuc-MCS-β72–167 mut GC Bx 2, the insert was homologous to −167 to −72 within the β1 promoter and had mutations in PGRE3, CAGCG ATCCAAAGCG CCCCTCTAGCG GCAGCGCCGCT CTCTTGTGCG GGCGCCGAC AATTACCGTTT CCCCTGGCGCC AGCGCATTTT GCAGCGCCGCA TTGGCG. For pLuc-MCS-β72–167 mut GC Bx 3, the insert was homologous to −167 to −72 within the β1 promoter and had mutations in PGRE3, CAGCG ATCCAAAGCG CCCCTCTAGCG GCAGCGCCGCT CTCTTGTGCG GGCGCCGAC AATTACCGTTT CCCCTGGCGCC AGCGCATTTT GCAGCGCCGCA TTGGCG. For pLuc-MCS-β72–167 mut GC Bx 4, the insert was homologous to −167 to −72 within the β1 promoter and had mutations in PGRE3, CAGCG ATCCAAAGCG CCCCTCTAGCG GCAGCGCCGCT CTCTTGTGCG GGCGCCGAC AATTACCGTTT CCCCTGGCGCC AGCGCATTTT GCAGCGCCGCA TTGGCG.
were similarly individually ligated into pLuc-MCS, creating the vectors pLuc-MCS-β421–456, pLuc-MCS-β211–240, and pLuc-MCS-β85–117, respectively. The composition of the recombinant pLuc-MCS vectors was confirmed by sequencing.

**Animal Cell Culture Conditions**

The basal medium for MDCK cells was DMEM/F-12 (50:50) supplemented with 15 mM HEPES (pH 7.4), 20 mM sodium bicarbonate, 92 U/ml penicillin, and 200 µg/ml streptomycin (DMEM/F-12). The basal medium was further supplemented with growth factors immediately before use (39). Stock MDCK cell cultures were grown in basal medium supplemented with 5 µg/ml bovine insulin, 5 µg/ml human transferrin, 5 × 10⁻¹¹ M triiodothyronine (T₃), 5 × 10⁻⁸ M hydrocortisone, 25 ng/ml PGE₁, and 5 × 10⁻⁸ M selenium (Medium K-1) in a humidified 5% CO₂-95% air environment at 37°C (39), and MDCK cells were routinely subcultured using EDTA/trypsin, as described previously (39). For experimental studies, MDCK cells were cultured in basal medium supplemented with 5 µg/ml insulin and 5 µg/ml transferrin. *Drosophila* SL2 cells were maintained in Schneider’s medium supplemented with 46 U/ml penicillin, 50 µg/ml streptomycin, and 10% heat-inactivated FBS in a humidified environment at 25°C. SL2 cells were detached from culture flasks by mechanical shaking.

**Transient Transfection Studies**

MDCK cells were transiently transfected using Lipofectamine, as previously described (37). To summarize, MDCK cells (plated at 10⁵ cells/35-mm dish) were cotransfected with 1 µg of the appropriate vector(s), as well as pSVβgal (0.2 µg). The next day, the medium was
changed, and 2 h later appropriate effector molecules were added. After an additional 4-h incubation, the monolayers were solubilized in reporter lysis buffer and centrifuged (14,000 rpm, 1 min). Drosophila SL2 cells (10⁶ cells/35-mm dish) were transiently transfected using Cellfectin with appropriate vectors (including pPAcGal) in 1 ml of Schneider’s medium lacking antibiotics and FBS. After the initial 4 h of transfection, FBS was added to a final concentration of 10%. The next day, an additional 1 ml of Schneider’s medium containing 10% FBS was added to the cultures. Twenty-four hours later, the medium was removed by aspiration and monolayers were solubilized in reporter lysis buffer.

The luciferase activity of cell lysates was determined using luciferase assay buffer [20 mM Tricine, 1.07 mM MgCO₃, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP]. Emitted light was measured in a Packard Tri-Carb 4530 Scintillation Counter with the coincidence circuit turned off. β-Galactosidase activity was determined using the Galacto-Star System. The β-galactosidase activity of experimental cultures was expressed as a fraction of the activity in control cultures.

Each luciferase assay determination was normalized with respect to its β-galactosidase activity. Each luciferase value was the mean ± SE of quadruplicate determinations. In each experimental set, the mean value was divided by the indicated control value to obtain the fold-stimulation/inhibition. The experimental results were then subjected to a one-way ANOVA and the Newman-Keuls multiple comparison test (Prism 4 software). Differences were significant when P < 0.05.

Preparation of Nuclear Extracts

Nuclear extracts were prepared from MDCK cells by a modification (1, 25) of the procedure of Dignam et al. (11). Confluent MDCK monolayers in 100-mm dishes were washed twice with PBS at 4°C, removed from culture dishes with a rubber policeman, and transferred into microcentrifuge tubes. After centrifugation (2,000 rpm, 10 s, 4°C), the pellet was resuspended in a hypotonic buffer [10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 μg/ml leupeptin] at 4°C (using ≥2X the packed cell volume). After swelling (4°C, 10 min), the material was vortexed (20 s) and centrifuged (2,000 rpm, 10 s, 4°C). The pellet was resuspended in a high-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.2 mM PMSF, 0.5 mM DTT, 0.5 μg/ml leupeptin). After 20 min at 4°C, the suspension was centrifuged (14,000 rpm, 2 min, 4°C), and aliquots of supernatant were quickly frozen in liquid nitrogen. Protein determinations were made using the Bradford method (3).

EMSAs

Synthetic double-stranded oligonucleotides were 3²P-labeled by random priming, using [γ-3²P]dCTP. Consensus CRE, mutant CRE, and consensus Sp1 oligos were 5’-end-labeled using [α-3²P]dCTP. Included among the oligonucleotides were 1) CTCTCCGGGC CGAGTCCCTGC CCCC TGCGGCC G (81 to −111, PGRE3); 2) GCTGCCTGCG CTGCCTCAC CACC (235 to −213, PGRE2); 3) GCGTCCGGCA GTAGTCCTCC CCCC (−445 to −432, PGRE1); 4) ATTTGCTGCG TGGTGTCC AAGCTAG (a consensus CRE); 5) ATTTGCTGCG TGGTGTCC AAGCTAG (a mutant CRE); and 6) ATTTGAGGC GCAGGGGGCCA GC (a consensus Sp1 site).

Nuclear extracts (2-6 μg) were first incubated in 9 μl binding buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, and 0.05 mg/ml poly(dI-dC) in either the presence or absence of unlabeled oligonucleotide (50–200-fold, 37°C, 10 min). Then, a 3²P-labeled probe was added, and the incubation continued (20 min, 37°C). In supershift experiments, antibody was either added simultaneously with the 3²P-labeled probe or following a 20-min incubation with the probe, as specified. The binding reaction was terminated by addition of gel loading buffer [25 mM Tris·HCl (pH 7.5), 0.02% bromphenol blue, 4% glycerol], and samples were separated on nondenaturing 4% acrylamide/0.001% bisacylamide gels at 35 mA. The gels were dried, subjected to autoradiography, and autoradiograms were scanned with a Bio-Rad scanning densitometer. Band intensities were quantified using the Quantity One program.

DNA Affinity Precipitation Studies

Confluent MDCK monolayers in 100-mm culture dishes were lysed at 4°C in 600 μl HKMG (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM DTT, 0.1% Nonidet P-40) containing 1 mM NaF, 1 mM Na₂VO₃, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM EDTA (6). Cells were sonicated (10 s, 4°C) and centrifuged (10,000 g, 5 min, 4°C, 2×). Cell extracts were incubated for 16 h with biotinylated double-stranded oligonucleotides (1 or 5 μg), including 1) 5’TGTCCGGGAG GAC TCCCTCCAC CAG (−432 to −454, PGRE1); 2) 5’-GCTGCTGCTG CGC GTGCCCTCAC CG (−213 to −235, PGRE2); and 3) 5’-CTCTCCGGGAC GAGTCCCTGC CGCTTG (−86 to −111, PGRE3). Control cell extracts were incubated either in the absence of oligo or with a biotinylated control oligo, 5’-CTACTGCTTAC TCTTGACTAC GAC (3). The results obtained with the control oligo indicated the specificity of binding. Biotinylated DNA-protein complexes were precipitated with streptavidin-agarose beads (1 h), washed with HKMG buffer (3×), separated by SDS-PAGE, and transferred to nitrocellulose. Western blots were analyzed using either anti-CREB, anti-Sp1, anti-Sp3, or anti-TBP antibodies (6, 30).

RESULTS

Role of PGRE1, PGRE2, and PGRE3 in PGE₁ Stimulation

Previously, we have shown that Na-K-ATPase β₁-subunit gene expression is regulated by PGE₁, 8-bromo-cAMP (8-BrCAMP), and phorbol 12-myristate 13-acetate (TPA) (25, 37). Our previous studies with 5’-deletion mutants indicated that the region between −83 and −182 within the human β₁-subunit promoter was sufficient to observe a PGE₁ stimulation. A putative CRE, AGTCCCTGTC, was identified within this region (−100 to −92). However, the 5’-deletion analysis did not exclude the possibility that other PGREs were also present in the human β₁-subunit promoter. Thus here the possibility is examined that additional PGREs are present in the β₁-subunit promoter, including a putative PGRE1 at position −445 to −438 (TGACCTTC) and PGRE2 at position −226 to −216 (GTTCCCTCA), in addition to the previously identified PGRE3 (PGRE3) at −100 to −92 (AGTCCCTGC) (Fig. 1A).

To determine whether these PGREs are individually functional, transient transfection studies were conducted with expression vectors containing each of these three specific elements, including pLuc-MCS β421–456, pLuc-MCS β211–240, and pLuc-MCS β85–117 (containing PGRE1, PGRE2, and PGRE3, respectively). The effect of 1.4 μM PGE₁, 1 mM 8-BrCAMP, and 1 mM TPA was examined in MDCK cells transiently transfected with these expression vectors. Figure 1B shows that PGE₁ and 8-BrCAMP caused equivalent stimulations, 100 ± 15% and 98 ± 12-fold, respectively, in transient transfections with pLuc-MCS β85–117 (containing PGRE3). TPA similarly caused a stimulation with pLuc-MCS β85–117, albeit to a lower extent (2.0 ± 0.8-fold, P < 0.05), indicating that the PKC pathway is also involved. In transient transfection studies with pLuc-MCS β421–456 (containing PGRE1) and pLuc-MCS β211–240 (containing PGRE2), significant stimu...
latory effects of PGE1 and 8-BrCAMP were also observed. However, with pLuc-MCS β421–456, the PGE1 stimulation (31 ± 13-fold vs. pLuc-MCS β421 controls) was lower than that obtained with pLuc-MCS β85–117 (100 ± 15-fold compared with pLuc-MCS β85–117 controls). A similar observation was made with regard to the 8-BrCAMP stimulation obtained with pLuc-MCS β421–456 (51 ± 8-fold vs. pLuc-MCS β421–456 controls), which was lower in magnitude than that obtained with pLuc MCS β85–117 under these conditions (98 ± 12-fold compared with pLuc MCS β85–117 controls). Luciferase activity was substantially lower with pLuc-MCS β211–240 (containing PGRE2); nevertheless, a significant PGE1 and 8-BrCAMP stimulation was observed relative to untreated pLuc-MCS β211–240 controls (30 ± 13- and 23 ± 2-fold, respectively).

Our previous 5'-deletion analysis indicated that stimulatory effects of PGE1, 8-BrCAMP, and TPA were retained in a 5'-deletion mutant (pHB83–182 Luc) containing PGRE3, but lacking PGRE1 and PGRE2 (25). The magnitude of the PGE1, 8-BrCAMP, and TPA stimulation did not differ from that obtained with pHB1–1141 Luc, the construct containing the entire β1-subunit promoter. To determine whether PGRE3 was required to obtain a PGE1 stimulation through the β1 promoter, we conducted transient transfection studies with pHB1–1141Luc (Δ72–299), with a deletion mutation, removing both PGRE2 and PGRE3 (illustrated by arrows in Fig. 1A), while retaining putative PGRE1.

Figure 1C shows a stimulatory effect of 1.4 μM PGE1, 1 mM 8-BrCAMP, and TPA in MDCK cells transiently transfected with pHB1–1141Luc (Δ72–299), as well as with pHB1–1141Luc. The total luciferase activity observed with PGE1, 8-BrCAMP, and TPA was higher with pHB1–1141Luc (Δ72–299) than with pHB1–1141Luc. However, when the stimulatory effects of PGE1, 8-BrCAMP, and TPA obtained in MDCK cells transfected with pHB1–1141Luc (Δ72–299) were compared with the level obtained in untreated control cells similarly transfected with pHB1–1141 Luc (Δ72–299), the observed stimulation by PGE1, 8-BrCAMP, and TPA (4.0 ± 0.4, 6.7 ± 1.3-, and 2.3 ± 0.3-fold, respectively), was no greater than the stimulation obtained in parallel cultures transfected with pHB1–1141Luc, relative to the pHB1–1141Luc control (6.1 ± 0.5-, 8.8 ± 0.4-, and 2.5 ± 0.2-fold for PGE1, 8-BrCAMP, and TPA, respectively).

**Binding of Nuclear CREB and Sp1 to PGRE1 and PGRE3**

**In Vitro**

**EMSA studies.** Previously, we conducted a number of studies including EMSAs, which indicated that both CREB and Sp1 bind to the region of the Pβ1 promoter region containing PGRE3 (25). EMSAs were similarly conducted to determine whether CREB and Sp1 also bind to the β1 promoter regions containing either PGRE1 or PGRE2. Synthetic 32P-labeled oligonucleotide probes utilized in these studies include 1) CTCTCGGGCC GAGTCCCTGC CCGCTGCCC CGC (–81 to –111), 2) GCTGGCTGGC CCTGCCCTAC CGC (–235 to –213), and 3) PGCCCGGCTA GCTGCCCTAC CCCAC (–456 to –432), which are homologous to regions on the β1 promoter containing PGRE3, PGRE2, and PGRE1, respectively. Following binding reactions with nuclear extracts and these labeled probes, the products were separated by PAGE and subjected to autoradiography (Fig. 2). The autoradiograms showed multiple 32P-labeled bands in each gel, each band presumably representing a unique nuclear protein-DNA complex.

**In previous EMSAs, we showed that an unlabeled consensus CRE oligo was able to compete against a 32P-labeled oligo containing PGRE3 for binding to nuclear proteins in MDCK cells. These results suggested that nuclear proteins such as CREB bind to this PGRE (25). A similar competition study was conducted to determine whether PGRE1 and PGRE2 could function in a similar manner. The effect of a 200-fold excess of unlabeled consensus CRE oligo (AGAGATTGGCCTGACGT- CAGAGAGCTAG) on the binding of 32P-labeled oligos (containing either PGRE1, PGRE2, or PGRE3) to nuclear proteins was examined (Fig. 2A).

The results of the competition study with excess unlabeled CRE oligo indicated that the intensity of a number of the 32P-labeled bands was reduced, primarily when 32P-labeled oligos containing either PGRE1 or PGRE3 were utilized. For example, when an excess of unlabeled CRE oligo was used, an 81% reduction in the intensity of band A was obtained with 32P-labeled GCTGCCTGGCC GACTCCCTAC CCCAC (containing PGRE1), and a 92% reduction in the intensity of band A' was obtained with 32P-labeled CTCTCGCC GCCTACCTTC CCGG (containing PGRE3). However, the ability of the unlabeled CRE oligo to compete with 32P-labeled GCTGCCTGGCC GCTTCCTAC CCC (containing PGRE2) was considerably lower (exemplified by a 49% reduction in the intensity of band A'). Similar results were obtained when the ability of an unlabeled consensus Sp1 oligonucleotide to compete with labeled oligos containing either PGRE1, PGRE2, or PGRE3 was examined, as shown in Fig. 2B.

Our previous EMSA results with a 32P-labeled PGRE3 oligo indicated that both an anti-CREB and anti-Sp1 antibody caused supershifts (25). Similar studies were conducted to determine whether an anti-CREB or anti-Sp1 antibody could supershift 32P-DNA-nuclear protein complexes that formed when either 32P-GCGTCCCGGA GTGACCTTC CCCAC (containing PGRE1) or 32P-GCTGCCTGGC GCTTCCTAC CCC (containing PGRE2) was used.

Figure 2C shows the supershifted bands that formed when EMSAs were conducted with an anti-CREB antibody and 32P-labeled GCGTCCCGGA GTGACCTTC CCCAC (containing PGRE1) and 32P-GCTGCCTGGC GCTTCCTAC CCC (containing PGRE2). However, a supershift with an anti-Sp1 antibody was only obtained when 32P-labeled GCGTCCCGGA GTGACCTTC CCCAC (containing PGRE1), rather than 32P-GCTGCCTGGC GCTTCCTAC CCC (containing PGRE2), was used. The specificity of the antibody reactions was indicated in control EMSAs conducted with either an anti-CREB or anti-Sp1 antibody as well as either 1) a labeled consensus CRE oligo (AGAGATTGGCCTGACGT- CAGAGAGCTAG), 2) a labeled mutant CRE oligo (AGAGATTGGCCTGACGT- CAGAGAGCTAG), or 3) a labeled consensus Sp1 oligo (ATTCAGTCGG GCGG- GGCGA GC) (Fig. 2D).

**DNA affinity precipitation studies.** To further investigate the ability of PGREs to bind transcription factors, DNA affinity precipitation assays were conducted. A biotinylated oligo, CTCTCGCCGC GACCTTCCTAC CCC (–86 to
Fig. 2. EMSAs with PGRE1, PGRE2, and PGRE3 oligos. A: EMSAs were conducted with 32P-labeled PGRE oligonucleotide probes complementary to either PGRE1, PGRE2, or PGRE3 (as indicated in MATERIALS AND METHODS) in both the presence and absence of a 200-fold excess of unlabeled CRE oligo. The band reduction obtained by the addition of a 200-fold excess of unlabeled consensus CRE was in the case of PGRE1 (81%, band A and 66%, band B). In the case of labeled PGRE2, 200-fold excess of the CRE oligo caused the band intensity to be reduced by 49 (band A‘), 36 (band B‘), and 48% (band C‘). Finally, in the case of labeled PGRE3, following the addition of excess unlabeled CRE oligo, the band intensity was reduced by 92 (band A“) and 55% (band B“). B: EMSAs were conducted with 32P-labeled PGRE oligonucleotide probes complementary to either PGRE1, PGRE2, or PGRE3 in both the presence and absence of a 200-fold excess of unlabeled Sp1 oligo. A reduction in the intensity of bands A, B, and C were observed with the addition of a 200-fold excess of Sp1 to the nuclear extract incubated with labeled PGRE1 (reduction by 96%, band A; 98%, band B; and 44%, band C), labeled PGRE2 (reduction by 28%, band A‘; 25%, band B‘; and 11%, band C‘), and labeled PGRE2 (reduction by 15%, band A‘; 13%, band B‘; and 16%, band C‘). C: supershifts were obtained by adding a rabbit polyclonal antibody against CREB to the binding reaction 20 min after the addition of 32P-labeled PGRE1 or PGRE2. D: supershifts were obtained by adding a rabbit polyclonal antibody against Sp1 to the binding reaction 20 min after the addition of 32P-labeled consensus CRE, mutant CRE, or consensus Sp1 oligo. Each experiment is representative of at least 3 determinations.

−111), which contained PGRE3, was incubated with an MDCK nuclear extract, followed by streptavidin-agarose precipitation, electrophoresis, and transfer of the nuclear proteins to nitrocellulose. The results of Western blot analysis (Fig. 3A) indicate that CREB, Sp1, Sp3, and TBP (a component of TFIIID) all coprecipitate with biotinylated CTCTCGGGCC GAGTCCCTGC CCCTGG, which contained PGRE3.

The ability of PGRE1 (TGACCTTC), PGRE2 (TGTCCTCA), and PGRE3 (AGTCCTCGTGC) to bind CREB and Sp1 was similarly assessed. The results of Western blot analysis (Fig. 3B) indicate that a 10-fold higher level of CREB binding was obtained with biotinylated 5‘-CTCTCGGGCC GAGTCCCTGC CCCTGG-3‘ (−86 to −111) containing PGRE3 than with biotinylated 5‘-GCTGCGCTGC CGTCCCTAC CGC-3‘ (−213 to −235) containing PGRE2. A threefold higher level of CREB binding was obtained with biotinylated 5‘-GTCGGGAGGT GACCTTCCC CAC-3‘ (−432 to −454) containing PGRE1 than the CREB binding obtained with biotinylated 5‘-GTCCTGGCG CGTCCCTAC CGC-3‘ (−213 to −235) containing PGRE2.

Similarly, 3.8-fold more Sp1 binding was obtained with biotinylated 5‘-CTCTCGGGCC GAGTCCCTGC CCCTGG-3‘ (−86 to −111) containing PGRE3 than with biotinylated 5‘-GCTGCGCTGC CGTCCCTAC CGC-3‘ (−213 to −235) containing PGRE2. Sp1 binding to biotinylated 5‘-GTCCCGGAGT GACCTTCCC CAC-3‘ (−432 to −454) containing PGRE1 was only 1.4-fold higher than the binding obtained with biotinylated 5‘-GCTGCGCTGC CGTCCCTAC CGC-3‘ (−213 to −235) containing PGRE2. These results are consistent with the EMSA results and the hypothesis that a nuclear protein complex containing CREB and Sp1 associates with PGRE1 and PGRE3 on the Na-K-ATPase β1-subunit promoter.

Functional Role of CREB and Sp1 in Regulation by PGRE1 and PGRE3

Because the results of our EMSAs and DNA precipitation assays suggested that CREB and Sp1 bind PGRE1 and PGRE3, the functional significance of these observations was evaluated. Initially, the effect of a dominant negative CREB (KCREB) on the PGE1 stimulation was examined. MDCK cells were transiently transfected with either pLuc-MCS β421–456 (which contains PGRE1) or pLuc-MCS β82–115 (that contains PGRE3), and KCREB, followed by a 36-h incubation to allow for KCREB expression. Figure 4A shows that when MDCK cells were cotransfected with KCREB and pLuc-MCS β421–456, or with KCREB and pLuc-MCS β82–115, the PGE1 stimulation was substantially reduced compared with untreated controls. In the case of pLuc-MCS β421–456, the PGE1

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stimulation was 3.8 ± 0.2-fold (following the extended incubation). In the presence of KCREB, luciferase gene expression was below basal levels observed with the empty vector, although a modest PGE1 stimulation was still observed relative to the KCREB control (to 1.6 ± 0.1-fold). Similar observations were made in the case of pLuc-MCS/82–115. In the absence of KCREB, a 5.1 ± 0.7-fold PGE1 stimulation was observed. In the presence of KCREB, the PGE1 stimulation was only observed relative to the PGE1 controls transfected with KCREB (1.4 ± 0.3). These observations are consistent with the involvement of CREB in mediating the PGE1 stimulation through PGRE1- and PGRE3-regulatory elements.

Dependence of Transcription on Sp1

To determine whether Sp1 is involved in regulating transcription through either PGRE1 or PGRE3, transient transfection studies were conducted with Drosophila SL2 cells (which

Fig. 3. DNA affinity precipitation of nuclear proteins. A: nuclear extracts from MDCK cells were incubated with a biotinylated PGRE3 oligonucleotide. Nuclear protein/oligo complexes were purified with streptavidin-agarose beads, separated on SDS/PAGE, and subjected to a Western blot analysis using either anti-CREB, anti-Sp1, anti-Sp3, or anti-TFIIID polyclonal antibodies. *, Specific band of interest; –, No Oligo; +, PGRE3. B: nuclear extracts from MDCK cells were incubated with biotinylated PGRE1, PGRE2, or PGRE3 oligonucleotide (as indicated in MATERIALS AND METHODS). Nuclear protein/oligo complexes were purified with streptavidin-agarose beads, separated by SDS/PAGE, and subjected to Western blot analysis using either anti-CREB or anti-Sp1 polyclonal antibodies. –, Specific band of interest. Experiments are representative of at least 3 determinations.

Fig. 4. Role of CREB in regulation. A: role of CREB. MDCK cells were transiently transfected either with pLuc MCS/421–456 and either Rc/RSV (empty vector; EV) or Rc/RSV-KCREB (KCREB) or pLuc-MCS/85–117 and either EV or KCREB. Subsequently, cultures were incubated 4 h in neither the presence or the absence of 1.4 μM PGE1. Luciferase activity was compared with the activity in the control condition (–PGE1, –T3). Results are representative of at least 3 independent experiments.

PGE1 stimulation observed with pLuc-MCS/85–117 by 60 ± 1%. Thus the inhibitory effect of T3 on the PGE1 stimulation could not necessarily be attributed to the binding of T3 to a TRE on the DNA.

Dependence of Transcription on Sp1

To determine whether Sp1 is involved in regulating transcription through either PGRE1 or PGRE3, transient transfection studies were conducted with Drosophila SL2 cells (which
are generally deficient in Sp proteins, including Sp1) (9). The expression vectors pLuc-MCS PGRE1 and pPacO, pLuc-MCS PGRE1 and pPacSp1, pLuc-MCS PGRE3 and pPacO, or pLuc-MCS PGRE3 and pPacSp1. The next day, the luciferase activity of the cultures was determined. The luciferase activity in experimental conditions was compared with the luciferase activity obtained with the control (either with pLuc-MCS PGRE1 and pPacO or pLuc-MCS PGRE3 and pPacO). Values are means ± SE of quadruplicate determinations.

The stimulatory effect of Sp1 on pLuc-MCS β421–456 and pLuc-MCS β85–117 gene expression in SL2 cells is presumably due to Sp1 binding to the GC boxes adjacent to the PGREs on these promoters. However, Sp1 binding to these GC boxes may also be required for PGRE function, which also affects transcription. These possibilities were further evaluated by means of transient transfection studies in SL2 cells with pLuc-MCS β72–167, containing the sequence illustrated in Fig. 6A, which includes PGRE3. In addition, Fig. 6A shows the sequences inserted into the other pLuc-MCS vectors utilized here, including −167 to −72 mut PGRE3 (contained within pLuc-MCS β72–167 mut PGRE3 and with mutations in PGRE3), and −167 to −72 mut GC box 1 (contained within pLuc-MCS β72–167 mut GC Bx1 and with mutations in GC box adjacent to PGRE3).

Figure 6B shows that when SL2 cells were cotransfected with pLuc-MCS β72–167 and pPacSp1, a 1,326 ± 192-fold increase in gene expression was obtained compared with control SL2 cells cotransfected with pLuc-MCS PGRE3 and pPacO. However, Fig. 6B also shows that the level of gene expression obtained with pLuc-MCS PGRE3 with a GC box mutation was only 6% of the level obtained with cotransfection with wild-type pLuc-MCS β72–167 and pPacSp1 (an 84 ± 13-fold stimulation was obtained).

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**Fig. 5. Role of Sp1.** SL2 cells were transiently transfected with either pLuc-MCS PGRE1 and pPacO, pLuc-MCS PGRE1 and pPacSp1, pLuc-MCS PGRE3 and pPacO, or pLuc-MCS PGRE3 and pPacSp1. The next day, the luciferase activity of the cultures was determined. The luciferase activity in experimental conditions was compared with the luciferase activity obtained with the control (either with pLuc-MCS PGRE1 and pPacO or pLuc-MCS PGRE3 and pPacO). Values are means ± SE of quadruplicate determinations.

**Fig. 6. Effects of mutations in PGRE3 in SL2 cells. A:** sequence of wild-type human Na⁺-K⁺-ATPase β1-subunit promoter from −167 to −72 (denoted PGRE3 WT) of the sequence from −176 to −72 with a mutation in PGRE3 (denoted mut PGRE3), the sequence from −167 to −72 with a mutation in GC box 1 (denoted PGRE GC mutant), and the sequence from −167 to −72 with the translocation of 2 GC boxes (denoted 2xGC trans mutant). **B:** SL2 cells were transiently transfected with either pLuc-MCS containing −167 to −72 in the β1-subunit promoter (pLuc-MCS PGRE3), pLuc-MCS −167 to −72 with a mutation in GC box 1 (pLuc-MCS PGRE3 GC mutant), or pLuc-MCS −176 to −72 with a PGRE3 mutation (pLuc-MCS PGRE3 mutant). The next day, the luciferase activity of the cultures was determined and compared with the luciferase activity obtained with pLuc-MCS −167 to −72 and pPacO. Values are averages ± SE of quadruplicate determinations.
Possibly, the stimulatory effect of pPacSp1 is simply due to Sp1 binding to the Sp1 site located at −111 to −117, independent of any interaction with the PGRE. However, Fig. 6B shows that in SL2 cells cotransfected with pPacSp1 and pLuc-MCS β72–167 mut PGRE3 (a vector with mutations in the PGRE3 site), the level of luciferase gene expression was substantially reduced compared with the wild-type phenotype. Although cotransfection of pLuc-MCS β72–167 mut PGRE3 with pPacSp1 still caused a 166 ± 27-fold increase relative to the pLuc-MCS β72–167 mut PGRE3 control (cotransfected with pPacO), this level of stimulation was only 13 ± 2% of the stimulation obtained with the normal genotype, pLuc-MCS β72–167, in the presence of pPacSp1.

**Effects of Mutations in Sp1 and PGRE3 on the PGE1 Response**

To study the role of PGRE3 and Sp1 in mediating the prostaglandin response, transient transfection studies were conducted with normal and mutant pLuc-MCS β72–167 vectors in MDCK cells. Figure 7A shows that both the PGRE3 mutation and the mutation in the adjacent GC box resulted in a complete loss of PGE1 stimulation. Figure 7A also shows that the same result was obtained when the two GC boxes immediately upstream of PGRE3 were translocated farther upstream (the translocation as illustrated in Fig. 6A). These results support the hypothesis that both PGRE3 and the adjacent GC boxes (located at −117 to −112 and at −143 to −139, respectively) are required for a prostaglandin response.

To study the effectiveness of PGRE3 relative to a consensus CRE, a study was conducted comparing the PGE1 response obtained with pLuc-MCS β72–167 and pCRE-Luc, a pLuc-MCS vector containing a minimal promoter with four consensus CRE sites (in tandem) immediately upstream from a TATA box. Figure 7B shows that equivalent PGE1 stimulation was obtained with pLuc-MCS β72–167 and pLuc-MCS CRE (31 ± 4- and 39 ± 9-fold, respectively).

**DISCUSSION**

The α- and β-subunits of the Na-K-ATPase are subject to differential regulation by a number of hormones and effector molecules (16, 24). The differential regulation of the α- and β-subunits can be explained, at least in part, by their coordinate regulation at the transcriptional level. External factors that primarily affect Na-K-ATPase β-subunit gene expression have received particular attention in those cases where the level of newly synthesized β-subunits is limiting to the formation of α/β heterodimers (15, 40). For example, in LLC-PK1 cells incubation in medium with low-K+ concentrations resulted in a selective increase in β-subunit mRNA levels, without a significant effect on α-subunit mRNA levels (22). Thus the consequent increase in Na-K-ATPase levels was attributed to the selective increase in β-subunits. However, the Na-K-ATPase β-subunit is not necessarily rate limiting for α/β assembly.

Indeed, changes in the external milieu result often result in changes in both α- and β-subunit mRNA levels. In MDCK

**Fig. 7. Effects of mutations in PGRE3 in MDCK cells. A:** MDCK cells were transiently transfected either with the vector pLuc-MCS, which contains a minimal promoter with a TATA box, pLuc-MCS containing −167 to −72 in the β1-subunit promoter (pLuc-MCS PGRE3), pLuc-MCS −167 to −72 with the 2X GC trans mutation (pLuc-MCS2xGCTrans), with pLuc-MCS −167 to −72 with a mutation in GC Box 1 (pLuc-MCSPGRE3GCmutant), or with pLuc-MCS −176 to −72 with a PGRE3 mutation (pLuc-MCSPGRE3mutant). **B:** transient transfection studies were conducted in MDCK cells with pLuc-MCS, pLuc-MCS CRE, and pLuc-MCS PGRE3. Transfected MDCK cells were treated for 4 h with either 1.4 μM PGE1 or untreated. Values for all the experiments are the mean luciferase activity (light units) ± SE of experiments performed in quadruplicate and normalized with respect to β-galactosidase activity. At least 3 experiments were performed. *P < 0.001, *P > 0.5 relative to pLuc-MCS control.
cells, low external K⁺ caused a 1.9-fold increase in α-subunit mRNA levels in addition to a 2.3-fold increase in β-subunit mRNA levels (2). Similarly, hyperoxia caused a 3.4-fold increase in β-subunit mRNA levels and a 1.4-fold increase in α-subunit levels in MDCK cells (43). Although veratridine caused a 1.6-fold increase in α-subunit mRNA levels in primary rat myoblasts, in addition to a 2.6-fold increase in β-subunit mRNA (36), the investigators nonetheless propose that the increase in β-subunit mRNA was responsible for the consequent increase in α/β heterodimer formation.

Nevertheless, different sets of stimuli can modulate α- and β-subunit levels in very different manners, even in a manner such that an increase in the α-subunit is the predominant change. Thus a detailed examination of both α- and β-subunit gene regulation may ultimately be required both in vitro and in vivo to obtain a full understanding of the phenomenon. Previously, we observed that PGE₁ and 8-BrAMP increase Na-K-ATPase β₁-subunit mRNA levels to a greater extent than β₁ mRNA levels (37). These changes were associated with an increase in Na-K-ATPase activity (37, 40). For this reason, we initiated our investigations with an analysis of Na-K-ATPase β₁-subunit gene expression in MDCK cells.

Previously, we presented evidence indicating that PGE₁ stimulates transcription of the human β₁-subunit gene and that regulation could be attributed, at least in part, to a PGRE, AGTCCCTGC (located at −92 to −100) (25, 37). In this study, we have presented evidence indicating that additional PGREs are involved in mediating the regulation of the Na-K-ATPase β₁-subunit gene by prostaglandins, including PGRE1 (TGACCTTC; located at −445 to −438); PGRE2 (GTC-CCTCA; located at −226 to −216); as well as the previously identified PGRE (AGTCCCTGC; located at −92 to −100, referred to in this report as PGRE3). Although the PGE₁ stimulation was also obtained with pLuc-MCS β211–240 (containing PGRE2), we have examined the regulation through the PGRE1 element in greater detail than PGRE2.

Our experimental results indicate that the PGE₁ response occurring through both PGRE1 and PGRE3 is dependent on CREB. Exposure to agonists that activate adenylate cyclase stimulates CREB phosphorylation (48). CREB phosphorylation may also occur in response to agonists that act through Ca²⁺ and/or PKC. A consequence of CREB phosphorylation at Ser 133 is the recruitment of CBP to the promoter region, and the binding of CBP to CREB (33). However, CREB phosphorylation is not a necessary indicator of target gene activation (48). In a number of promoter systems, CREB binding to a single CRE site is not sufficient to mediate a significant functional response to cAMP (34). In these cases, a strong regulatory effect of CREB is observed only if multiple CREB binding sites are present (35), or if additional regulatory elements are present. Included among such regulatory elements are the hepatic nuclear factor 4 binding site in the tyrosine aminotransferase promoter (32) and C/EBP in the phospholipidate carboxykinase promoter (34). Thus a number of investigators have concluded that CREB requires additional regulatory partners, including Sp1, to recruit the transcriptional apparatus to the promoter region of CREB-activated genes (48).

Our results also indicate that Sp1 is involved in mediating the effects of PGE₁ on Na-K-ATPase β₁-subunit gene transcription. Previously, Sp1 was reported to play a critical role in the upregulation of the Na-K-ATPase β₁-subunit gene. However, in this case the Sp1 binding site was different (located at −59 on the rat promoter) and was sufficient for the increased transcription (which occurred in response to hyperoxia) (42). An Sp1-Sp3 interaction was proposed to occur in this case, rather than an Sp1-CREB interaction. In contrast, synergism between an ATF/CRE site and an adjacent downstream GC box was proposed to be required for basal transcription of the rat Na-K-ATPase α₁-subunit gene (21). Regulatory interactions between transcription factors observed under basal conditions may be altered during the upregulation of transcription. For example, basal transcription of the folic receptor type β gene depends on a synergistic interaction between Sp1 and ets, as well as repression by upstream AP-1 like elements (17). Upregulation (which occurs in response to all trans-retinoic acid (RA)) involves the binding of RA receptor α (RARα) to the Sp1 site and reduced association of RARβ and γ to the AP-1 site. Similarly, under basal conditions the transcription of the plasminogen activator inhibitor-1 gene is minimal in vascular smooth muscle cells, due to a binding complex between a transcriptional repressor and Sp1 (which involves 2 Sp1 sites) (7). Upregulation of the plasminogen activator inhibitor-1 gene by glucose in vascular smooth muscle cells involves the release of the transcriptional repressor from this complex.

Our DNA affinity precipitation studies indicate that Sp3, like Sp1, binds to PGRE3 in the human Na-K-ATPase β₁ promoter. Sp3 may act as either a transcriptional repressor or a transcriptional activator. While Sp3 represses Sp1 transcriptional activation of the human thrombin receptor (46), Sp3 has been observed to upregulate Sp1 transcriptional activation of the hepatic growth factor promoter (47). Further investigations are needed to evaluate whether Sp3 acts as either an activator or a repressor in mediating regulation through PGREs.

The TR was previously reported as being a CREB binding partner and regulating transcription of the Na-K-ATPase β₁ promoter (14). In GH4C1 rat pituitary cells, the TR was observed to antagonize CREB-mediated transcription of the pituitary-specific transcription factor GHF-1/Pit-1 (28). CREB binds to two CREs in the GHF-1/Pit-1 promoter. Following treatment with a cAMP agonist, CREB is phosphorylated by cAMP-dependent protein kinase, while binding to two CRE sites. Although the TR does not bind to the GHF-1/Pit-1 promoter, an interaction of the TR with CREB prevents CREB phosphorylation and gene activation. Similarly, in the human prepro TRH promoter, the TR can bind to four different TREs, including two TRES with overlapping TRE/CRE bases (44). In this case, the TR-T3 complex was similarly inhibitory to cAMP-mediated regulation, while unliganded TR was stimulatory. In this report, T₃ antagonized the PGE₁ stimulation observed with pLuc MCS β421–526 (which contains PGRE1). PGRE1 (−438 to −445) is overlapping with the TRE in this region. However, T₃ also antagonized the PGE₁ stimulation obtained with pLuc MCS β85–117 (which contains PGRE3), suggesting that the inhibitory effect of T₃ is not necessarily the result of a TR-T3 complex binding to a TRE.

Here, we have observed that the PGE₁ stimulation obtained with pLuc-MCS β72–167 (that contains PGRE3) and pLuc-MCS β421–526 (that contains PGRE3) is of a much greater magnitude than that with pHβ1–1141Luc. Possibly, additional (but as of yet undefined) regulatory elements are present on the entire Na-K-ATPase β₁-subunit promoter that modulate the
amplitude of the PGE$_1$ response. Other regulatory elements on the β$_1$ promoter, which may play a role in mediating prosta-
glandin’s effects, include a CAAT box, several Sp1 sites, a CREB binding site, and a TATA box, which are clustered close
to the initiation site for transcription in the Na-K-ATPase β$_1$-subunit promoter, in addition to other elements more distal
from the transcription initiation start site. Thus the ability of either PGRE1 and/or PGRE3 to mediate a prostaglandin re-
sponse is dependent on the complexities of the local hormonal milieu (as exemplified by the case of thyroid hormone and
PGRE1). Future studies will be concerned with delineating the nature of these complex hormonal interactions.

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