Identification of a Novel Set of Genes Regulated by a Unique LXRα-Mediated Transcription Mechanism

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SUMMARY

We have reported previously that LXRα can mediate a novel cAMP dependent increase in renin and c-myc gene transcription by binding as a monomer to a unique regulatory element, termed the CNRE. To determine if this novel action of LXRα has global implications on gene regulation, we employed expression profiling to identify other genes regulated by this unique mechanism. Here we report the existence of a set of known and unknown transcripts regulated in parallel with renin. Querying the Celera Mouse Genome Assembly revealed that a majority of these genes contained the consensus CNRE. We have confirmed the functionality of these CNREs by competition for LXRα binding via EMSA assays, and by the use of CNRE decoy molecules documenting the abolishment of the cAMP-mediated gene induction. Taken together, these results demonstrate that the interaction between cAMP-activated LXRα and the CNRE enhancer element is responsible for widespread changes in gene expression and identify a set of LXRα/cAMP regulated genes that may have important biological implications.

INTRODUCTION

The transcription factor Liver X receptor-alpha (LXRα), a member of the nuclear hormone receptor superfamily, regulates the expression of genes involved in cholesterol homeostasis and bile acid synthesis (1,2). The best-known mechanism of LXRα mediated transcriptional activation occurs through interactions with compounds such as oxysterols (22-cho) or retinoic acid (9cRA) and results in heterodimerization to other transcription factors including RXRα, and PPARγ (3). Transcription of target genes, such as the cholesterol 7α-hydroxylase gene, occurs through a classical DR4/LXRE (5'-GGTTTAAATAAGTTCA-3') response element (4).

The aspartyl protease renin is synthesized in the kidney and secreted into the plasma. It is the rate limiting enzyme in angiotensin biosynthesis, and thus, plays an important role in blood pressure and volume regulation. Renin gene expression and secretion is mediated, in part, by intracellular levels of cAMP in kidney juxtaglomerular (JG) cells (5). Previously, we have identified a cAMP responsive element, distinct from the classical cAMP responsive element, in the promoter region of the mouse renin gene and have termed this element CNRE (5'-*TACCTAACTTGGCTCACAGGCAGAATTTATC*-3') (6). Homologues of this element, found at position -619 to -588 of the mouse *Ren-1*^{D} gene, have been found in the mouse *Ren-1*^{C} and *Ren-2* genes as well as in the rat and human renin genes (7). Furthermore, using a yeast one-hybrid screening approach, we demonstrated that LXRα bound to this sequence while functional studies using promoter/reporter gene chimeric constructs revealed that LXRα increased basal levels of renin expression and mediated the cAMP-dependent induction of mouse and human renin gene expression (8).

Interestingly, the previously reported LXRα ligands, such as 22-cho, had no effect on renin gene expression nor on expression of renin gene promoter/reporter gene chimeric constructs. Moreover, studies conducted with N-terminal deletion mutants of LXRα indicated that the binding mechanism and positive regulation of these genes through the CNRE element uniquely occurred as a monomer, in contrast to the action at the classical LXRα/DR4 element. Further studies revealed that the c-myc gene, which also contains the CNRE element, was similarly regulated (9). The discovery of LXRα as a regulator of renin gene expression by a previously unknown mechanism lead to the global question as to whether a set of genes exists that could be regulated in response to cAMP by LXRα. To address this question, we performed expression profiling of a mouse kidney juxtaglomerular

cell line (8) stably transduced with green fluorescent protein (10) or murine LXRα plus GFP and treated with cAMP or vehicle.

EXPERIMENTAL PROCEDURES

Mouse Kidney As4.1 Cell Culture and cAMP Stimulation.

The mouse renin-expressing cell line, As4.1 (ATCC CRL2193), were previously isolated from the kidneys of transgenic animals harboring a chimeric renin gene promoter/SV40 T antigen construct (11). Cells were cultured in high-glucose DME (Gibco Life Sciences) supplemented with 10% fetal calf serum (FCS) and antibiotics at 37°c in 5% CO_2. Stable cell lines infected with a bicistronic retroviral construct expressing either GFP alone or GFP plus mouse LXRα were generated as previously described (12). For cAMP stimulation, cells were first made quiescent in DME containing 0.1% FCS for 12hrs. Fresh media was added containing DME + 0.1% FCS plus either vehicle (PBS) or 1mM 8-Br-cAMP and incubated for various lengths of time. Total cell RNA was harvested using the TRIZOL reagent and was assessed for quality by spectrophotometric and electrophoretic analysis.

Construction of the 19,064 Element Mouse cDNA Microarray

The cDNA microarrays generated in our laboratory consisted of 19,064 elements that included 2,432 cDNAs from three mouse libraries donated by colleagues at the Brigham and Women's Hospital (David Beier, neonatal kidney and brain library; CC Liew, nucleated erythroid cell library. Genes and ESTs were identified using sequence comparison of the mouse Unigene EST database. An additional 15,264 cDNA clones, derived from the NIA15k mouse developmental set, were spotted resulting in a total of 18,296 cDNA clones available for expression analysis (13). For use in the determination of non-specific hybridization, cDNAs corresponding to several bacterial genes were also spotted in various regions of the array for a total of 768 control elements, thus resulting in the generation of a 19,064 element cDNA array.

Amplified cDNA inserts were obtained from purified plasmid DNA templates, lambda-phage clones or directly from bacteria harboring plasmids containing cDNA inserts. Briefly, purified plasmids were amplified using T7 (5'-GTAATACGACTCACTATAGGGC-3') and T3 (5'-AATTAACCCTCACTAAAGGG-3') or SP6 (5'-ATTAGGTGACACTATAG-3') primers with the cycling parameters of 94°c for 1min, 37°c for 1min, and 73°c for 1min 30sec for 40 cycles, with an additional extension cycle of 73°c for 8min. Libraries consisting of bacteria or phage received an initial incubation cycle of 94°c for 5min before entering the previously mentioned cycling

parameters. Amplified clones were analyzed for single-band product by agarose gel electrophoresis. PCR products were purified by ethanol precipitation in 96-well format as previously described (14). Purified inserts were spotted in linear format at 250μ center-to-center distance on CMT-GAPS (Corning, New York) slides using the GMS417 Arrayer (Affymetrix).

Probe Labeling and Hybridization

Total RNA (70μg) was used as template to generate fluorescently labeled cDNA probes by a single round of oligo dT primed reverse transcription in the presence of either Cy3-dUTP or Cy5-dUTP. First strand cDNA derived from RNA extracted from As4.1 cells that were transduced with the GFP retrovirus and treated with vehicle was used as reference cDNA in all experiments and was labeled with Cy3 while cDNA derived from RNA isolated from cells treated with cAMP and/or transduced with LXRα was labeled with Cy5. The labeled probes were purified using G50 spin columns (Pharmacia Biotech), combined and resuspended in a hybridization buffer containing 50% formamide and hybridized to the array in the presence of blocking DNAs (mouse cot-1 genomic DNA, oligo-dA, tRNA) for 16 hrs at 42°c under a coverslip. After hybridization, slides were washed in 1%SSC plus 0.1%SDS to remove the coverslip and remove non-hybridized probe and then sequentially in 0.5%SSC followed by 0.1%SSC. The slides were dried by centrifugation at 2000rpm for 2min and were immediately scanned and Cy3/Cy5 signal intensities were measured using a GMS418 Scanner (Affymetrix).

Microarray Data Collection and Analysis

The intensities Cy3 and Cy5 fluorescence of each spot were measured by overlaying a quantitation grid over the scanned image (ScanAlyze). Local background was subtracted from the overall spot intensity in both channels. Nonspecific cross-hybridization signal was also determined. Signals from each bacterial clone were measured, the values averaged and subtracted from the murine cDNA spots within that sub-array. Spots that did not meet a value > 1.5x background in either channel, at all experimental time points, were flagged or excluded. Calculated pixel-by-pixel correlation coefficients (*Ch1GTB2* and *Ch2GTB2*) for Cy3 and Cy5 fluorescent intensities in each spot was used to determine overall spot quality and spots which contained values of < 0.6 in both channels were also flagged or excluded.

For total array conditional comparisons, ratios (Cy5/Cy3) for each slide were calculated and to account for differences in overall hybridization and labeling efficiencies ratios were $\log_2$ transformed and median centered. The resulting data was then normalized using a 'lowess-fit' intensity-dependent algorithm and subjected to significance filtering software, based on observed vs. expected values (15,16). All conditions were combined in one large dataset and hierarchical clustering, using Pearson centered correlative and complete linkage-clustering algorithms, were then employed to generate a tree containing gene expression clusters with similar temporal expression profiles in all conditions using GeneSpring Software, version 4.13 (SI Genetics). A subset of genes was then obtained which contained an expression profile similar to mouse renin for subsequent analysis.

Multiplex RT-PCR

A semi-quantitative reverse transcriptase PCR (RT-PCR) was employed for validation as follows. Gene-specific primers were generated based on the known EST sequence. SpliceView and ExonPCR programs were utilized to generate primers which had a high probability of spanning an intron (17,18). Primers specific for 18s RNA (Forward 5'-CGG CTA CAT CCA AGG AA-3'; Reverse 5'-GCT GGA ATT ACC GCG GCT-3') were added as an internal positive control. Template RNA (500ng), gene-specific primers (20pM), 18s RNA primers (20pM), Oligo dT (500ng) were added to a RT-PCR kit (Amersham Pharmacia) and reactions were subjected to low-cycle PCR as follows; first-strand synthesis (1 cycle of 60°c for 30mins, 94°c for 2mins), amplification (18-25 cycles of 94°c 1min, 55°c 1min, 72°c 1min), extension cycle (1 cycle of 72°c 5min). Aliquots of the resultant products (5μl) were subjected to 2% agarose gel electrophoresis for further analysis. All RT-PCR experiments were conducted in triplicate using at least three separate RNA samples.

Double-stranded Decoy Experiments and Electrophoretic Mobility Shift Assays (EMSA)

A stock solution (150μM) of complementary phosphorothiate-modified single stranded oligomers (Invitrogen) were incubated at 65°c for 10mins and allowed to anneal at 25°c for 2hours in annealing buffer (100mM Tris-HCL pH 7.5, 1M NaCl, 10mM EDTA in DEPC-treated water). For experiments, annealed oligomers were diluted to various concentrations using annealing buffer while excess stock was stored at -20°c for future use.

Assessment of decoy transduction efficiency was conducted as previously reported with some adjustments

(19). As4.1 cells were grown to 70% confluence and transduced with 5-100μM of FITC labeled double-stranded decoy oligonucleotides containing the following sequences from previously published data (20): CNRE decoy (sense 5'-*TAC CTA ACT TGG TCT CAC AGG CTA GAA TTT ATC*-3'), CRE decoy (sense 5'-*GCT TAC CCA CAG TCC CCC GTG ACG TCA CCC GGC*-3'), Scrambled DNA (5'-*GTC AGC TAG TGT TGA CAG GCC AGT TAG GTC TCG AG*-3') using oligofectamine reagent (Invitrogen). After 48 hours, detection of positively transduced cells was conducted by fluorescent microscopy using a conventional fluorescein detection filter. Untransduced cells and phase contrast microscopy were utilized as a control.

For assessment of decoy effect on target gene transcription, As 4.1 cells, containing either GFP or LXRα were transduced with 40mM of non-FITC labeled decoy as previously mentioned. After 24 hours, cells were washed with phosphate buffered saline (PBS) then grown in serum-reduced media (DME + 0.1% FBS) for 12hours. Next, fresh growth media was added containing 0.1% FBS (Gibco BRL) and either 10mM 8-Br-cAMP or PBS (vehicle), and allowed to incubate at 37°c in 5% CO_2 for a period of six hours. After treatment, total RNA was isolated using Trizol (Invitrogen) and an aliquot subjected to quality assessment by spectrophometric and agarose gel analysis.

EMSA assays were performed as described previously with some modifications (7). As4.1 cells were serum restricted and treated with 1mM 8-Br-cAMP or vehicle as previously described. Nuclear protein extracts were prepared using the NuclearPrep Kit (Pierce Biolabs) and protein concentration assessed by spectrophotometric assay. Nuclear extracts were then aliquoted and stored at -80ºc for future use. For competition experiments, 10μg of extract from As4.1/LXRα was incubated with 1.57pM γ^{32}P-labeled probe containing the ren-1^{d} CNRE sequence (5'-CTA ACT TGG TCT CAC AGG CTA GAA-3') and 100-fold molar excess of unlabeled probe containing the CNRE and flanking 6bp sequences from each identified gene. After incubation at 37ºc for 30mins, the reaction was loaded onto a 6% polyacrylamide gel and electrophoresed in 1x Tris-Borate EDTA buffer for 2hours @ 200v. After electrophoresis, the gel was dried and exposed to autoradiography film overnight at -80ºc. For antibody neutralization assays, the reaction was carried out as previously described, however after incubation with lysate reactions were further incubated with either anti-goat IgG (Santa-Cruz Biotech), anti-LXRα (Santa-Cruz Biotech), or gelshift buffer for an additional 15mins.

RESULTS

Global Gene Expression is Affected by LXRα in As4.1 JG Cell Cultures

The novel action of LXRα in the regulation of renin and c-myc genes leads us to ask if other genes are regulated in a similar manner. To address this, we initiated a cDNA-based transcript profiling study. A mouse cDNA microarray was generated which contained total of 19,064 spotted elements. Table1 indicates the results of querying the cDNA sequences against the Unigene database (build# 88) which revealed 6,446 (35.2%) cDNAs matched to known genes and 5,990 (32.7%) matched to other reported ESTs, resulting in a total 12,436 (67.9%) of previously reported sequences. An additional 5,910 (32.3%) were unable to be matched, and thus, are considered novel. Additionally, 768 (4.2%) bacterial control spots were distributed throughout the array as control elements for normalization.

Serum restricted mouse kidney juxtaglomerular cells (As4.1), stably transduced with either GFP plus mLXRα (As4.1/LXRα), or GFP (As4.1/GFP), were treated with either 8-Br-cAMP or PBS (vehicle) for 1-24 hours (1, 6, 12, or 24hrs). Total RNA was used as template to generate fluorescently labeled cDNA probes by a single round of oligo-dT primed reverse transcription in the presence of either Cy3-dUTP or Cy5-dUTP. Standard background subtraction and normalization protocols were then employed and clustering analysis was performed (Figure 1). These results indicate that the greatest amount of differential gene expression occurred in cells expressing LXRα, in the presence of cAMP, when compared to all other conditions, and thus, demonstrate our previous observations of LXRα as a cAMP responsive transcription factor.

Identification of LXRα Modulated Transcripts that Exhibit a Ren-1^d Expression Profile.

We next asked if there existed a subset of genes with an expression pattern similar to that observed for renin. Utilizing the temporal expression profile of mouse *Ren-1^d*, where expression peaks at 6 hrs (Figure 2A and 2B), to query the entire set of expressed genes with a correlation coefficient cutoff of ≥0.92, a subset of genes were then obtained for subsequent cluster analysis (Figure 3A and 3B). Table 2 indicates a list of 41 genes that exhibit an increase in transcriptional activity, peaking at 6hrs. The gene descriptions in this table suggest that LXRα is able to modulate the expression of genes with diverse functional properties. These results indicate that, at 6 hours, LXRα+cAMP is able to induce the expression of these genes, with an observed range of induction of 3.45 to 51.7

fold, with 38 (92.6%) genes at a $p \leq 0.05$, and a subset of 24(58.5%) genes at a $p \leq 0.01$ when compared to all other conditions. The average fold induction (Figure 3D) in LXRα+cAMP treated cells, at the 6hr timepoint, was 8.38 fold higher when compared to all other conditions.

Identification of Genes that Contain an Upstream CNRE Element and RT-PCR Validation.

We next asked if these genes identified, by virtue of having an expression profile similar to renin, contained a CNRE element. The EST sequences corresponding to each of the 41 genes was compared to the Celera Mouse Genome Assembly to obtain the surrounding genomic sequence for each gene. Fifty kilobases (50Kb) of flanking sequence was queried for the presence of the consensus CNRE element [*5'-TNN(**T/G**)TC(**C/T**)CA(**C/G**)AGG-3'*]. Since most the ESTs to be queried are not matched to known genes, and are thus considered novel, a 50Kb distance was utilized to ensure the probability of analyzing a promoter region. The 5' end of the EST sequence was used as an 'anchor' and all consensus CNRE hits are reported in terms of the distance to their respective anchors. The results of this search indicated 16 genes (Table 3) that contain a consensus CNRE within 50Kb of the anchor. The total number of genes could further be divided into groups that contain CNRE elements within a short (0-15kb; 7 genes), moderate (16-30kb; 9 genes), and long distance (31-50kb; 7 genes) from the anchor sequence.

RT-PCR assays were conducted to validate temporal expression profile results by using EST-specific primers generated with a high possibly of spanning an intron using splice prediction software (SpliceView and ExonPCR). An internal control set of primers, generated to amplify 18s RNA, was added to each reaction to account for initial template concentration and all amplification reactions were conducted under low-cycle number conditions. The results from these experiments (Figure 4) indicate 15 out of 16 of genes that contained a CNRE were validated by a marked increase in amplified product present in cells transduced with LXRα and treated with cAMP, when compared to cells treated with vehicle or cells transduced with GFP and/or treated cAMP for a period of six hours.

Effects of Oligonucleotide Decoy Molecules on cAMP Induced Gene Expression in Mouse As4.1Cells

To determine whether the temporal expression profile exhibited by the identified genes was the result of LXRα directly modulating transcription levels via binding a CNRE enhancer element within the promoter region, or

through some other indirect mechanism, we employed a decoy strategy. Double-stranded decoy molecules that correspond to the CNRE element present in the mouse renin (Ren-1^d) promoter were generated to determine whether this decoy would be able to 'sequester' endogenous LXRα , and hence, suppress target gene inducibility. Control double stranded DNA representing decoy to the classical cAMP-response element (CRE) binding protein (CREB) enhancer sequence, and a scrambled DNA containing the CNRE nucleotides were utilized to assess specificity of the results. FITC-labeled decoy was used to analyze the transduction efficiency in As4.1 cells. Various concentrations (0-100μM) of DNA molecules were utilized in order to determine an optimal concentration that would yield high transduction efficiency, with low cytotoxicity. Efficiency was assessed by the percentage of positive nuclei under a fluorescent microscope after 24 hours. The results indicated 40μM to be the sufficient for 100% cellular transduction, with no visible cytotoxicity. Figure 5 indicates all three DNA molecules exhibited a similar efficiency when employed at 40μM. No visible fluorescence was observed in cells that were mock transduced.

As.4.1 cells, transduced with either LXRα or GFP were transduced with either the CNRE decoy, CRE decoy, or CNRE scrambled DNA. After a period of twenty four hours, all cells were serum restricted for twelve hours and then treated with vehicle or 8-Br-cAMP for six hours. Total RNA was harvested and RT-PCR was conducted using gene specific primers as mentioned before. RT-PCR assays conducted with mouse Ren-1^d, in the presence of CNRE decoy or control DNAs, indicate that the CNRE decoy is indeed able to inhibit LXRα-mediated induction of transcription (Figure 6A). However, no inhibition of Ren-1^d gene expression was observed when cells were treated with the classical CREB protein binding element (CRE) decoy or with a molecule containing the scrambled CNRE sequence. Results from RT-PCR experiments, using primers specific for genes which contained a CNRE element (Figure 6B) revealed that 11 out of 16 genes whose cAMP inducible expression was suppressed specifically by the CNRE decoy, suggesting that these genes are regulated by LXRα and cAMP through a cis-element CNRE binding sequence located in close proximity to the target gene.

Assessment of Direct Interaction of LXRα to Celera Identified CNRE Elements

We utilized electrophoretic mobility shift assays (EMSA) to determine if LXRα could directly interact with the identified CNRE elements present in genes affected by decoy molecules. Nuclear extracts were prepared from

As4.1/LXRα or As4.1/GFP cells which were treated with 8-Br-cAMP for 6hours. Double-stranded EMSA probe(s) for each gene were generated which contained the Celera identified CNRE sequence, with an additional 6 nucleotides of flanking sequence, to assess direct LXRα interaction. Competition assays were conducted using a double-stranded ^{32}P-labeled oligomer probe, containing the CNRE element from mouse renin (*ren-1^{d}*) in the presence of 100-fold molar excess of each unlabeled probe containing the CNRE sequence present in each specific gene. Results in Figure 7A demonstrate that the CNRE elements present in 9 out of the 11 decoy affected genes queried were able to effectively compete the labeled renin CNRE probe for LXRα binding as evidenced by abolishment in signal. No competition was observed from the CNREs in the remaining 5 out of the total 16 genes assayed.

To determine if the competition observed was LXRα specific, we employed a LXRα specific antibody (P-19) to neutralize binding of CNRE/LXRα direct interactions. Labeled probes, containing either the cis-element CNRE sequence from *ren-1^{d}, Placental specific homeobox (psx-1), or KIA0877* genes were incubated with As4.1/LXRα or As4.1/GFP nuclear extracts in the presence of anti-LXRα antibody. Figure 7B indicates a strong shift is observed in As4.1/LXRα control (-Ab, or + goat IgG) cell lysates with the *ren-1^{d}*, or *psx-1* probes when compared to the *KIAA0877* probe. However, in the presence of anti-LXRα antibody the CNRE/LXRα interaction is abolished from the *ren-1^{d}* probe. Likewise, reduced binding, but not complete abolishment, is also observed between the *psx-1*/CNRE probe. No LXRα shift was observed in control extracts (As4.1/GFP) or extracts incubated with KIA0877 probe and/or antibody.

DISCUSSION

Nuclear orphan receptor-mediated transcriptional regulation can be divided into two major sequences of cellular events. First, the inactivated nuclear receptor becomes activated by a conformational change, induced either by ligand binding or phosphorylation at key sites within the receptor (21). Second, activated nuclear receptor is then translocated to the nucleus to regulate target gene transcription either as a monomer or complexed with other transcription modulatory proteins (10). Previously, we identified LXRα as one of the key modulatory proteins that control the expression of renin in both cultured mouse kidney juxtaglomerular (As4.1) (8), and human lung (Calu-6) cells (22), both of which were transduced with an LXRα, or GFP, expression vector. Specifically LXRα, in the presence of cAMP, upregulates renin gene expression by directly binding as a monomer to a unique cAMP/negative response element (CNRE) located in close proximity to the transcriptional start site. Furthermore, we also demonstrated the c-MYC was regulated in a similar manner. Taken together, the previous data suggested that cAMP-activated LXRα could transcriptionally regulate genes with various cellular functions and implies a more ubiquitous role of LXRα/CNRE-mediated gene regulation in cellular physiology. Indeed, the results of this current study strongly support this contention.

The clones spotted on the mouse 19k array were generated from various cDNA libraries that consisted of cDNA clones from mouse brain, kidney, and erythroid blood cell libraries. The functional distribution of known genes on the array correlated with the expected division of expressed protein functions involved in normal cellular physiology and indicates that, although the various clones were obtained from different sources, the cumulative clone set maintained a representative functional allocation with respect to cellular physiology (23). Moreover, since a moderate number of spotted clones were unable to be matched in the Unigene database, and thus are considered novel, our mouse array provides a useful gene discovery source with which to identify novel genes that are regulated by LXRα in the presence of small molecule activators such as cAMP. It is certain that since the human and mouse genomes are now sequenced, and the era of functional genomics is becoming fully realized, more of these novel genes will eventually become known and functionally annotated and will provide further information as to the role of LXRα in global gene regulation and the implications to cellular physiology (24-26).

Our previous reports demonstrate that in cells overexpressing LXRα, renin mRNA levels increase as early as one hour in response to cAMP that peaks at six hours (8). This expression ‘signature’ of mouse renin was also

confirmed by our RT-PCR results in this study. To identify genes that are potentially regulated by activated LXRα, we queried the entire dataset of expressed genes for genes that exhibited an expression profile similar to that of renin. Some of the listed genes, such as *Hsc70, P450*, and *Ufd1* have been reported to be involved in the signal transduction pathway of nuclear receptor inactivation, and activation, respectively while the P450 family of genes has been reported to be transcriptionally regulated by various nuclear receptors (27-29). Another subgroup of genes from this list (*Psx-1, MM-1, and OxyR*) have been reported to be involved in modulating gene transcription by directly interacting with the promoter regions of other target genes (30-32). It should be noted that the intracellular levels of cAMP and LXRα transcript in these cells is markedly elevated and hence probably result in a non-physiologically high levels of activated LXRα. Thus some of the genes identified, although demonstrate a significant temporal change in transcript abundance *in vitro*, may not change in a temporal manner by conventional detection methods *in vivo*. It will be interesting to compare the changes observed *in vitro* under a more physiologically relevant condition, however, it is not within the scope of this communication.

We used a conservative algorithm to identify genes that contained a consensus CNRE binding sequence (33). This strategy would allow us to identify genes that contained this enhancer motif within a 50kb region upstream of the transcriptional start site since previous reports indicated the CNRE element as being located upstream of other identified genes. The results of the analysis for genes containing a CNRE enhancer element within this subset indicated yet a smaller subset of genes that contained a sequence that matched the consensus LXRα binding site. Not all of the genes exhibiting a profile of expression similar to that of renin contained a CNRE enhancer. Several possible explanations exist for this finding. One likely explanation is that we have not eliminated secondary effects of LXRα activation, i.e., the induction of expression of a transcription factor or factors by LXRα that then induces the expression of other genes secondary to the initial actions of LXRα. Indeed, inspection of the genes induced by LXRα or cAMP activated LXRα reveal several known or putative transcription factors. Furthermore, while we have searched the 50KB upstream of the EST with our best estimate of a consensus CNRE, it is possible that other sequences that deviate from the consensus sequence are functional but ignored in the bioinformatic screening process. Moreover, it is likely that LXRα binds and exerts action through other response elements distinct from the CNRE, such as the previously described DR4 sequence (34). However, it is important to note that while not all of the genes that possessed an expression profile similar to that of renin was shown to have a

CNRE in the 50KB region, of those that did, a vast majority (15/16) had profiles of expression that were verified by an independent assay (RT-PCR) to be similar to renin.

Although the majority of genes containing the CNRE exhibited reduced expression levels in the response to CNRE decoy administration, a few genes remained unaffected. Perhaps the CNRE sequence, found in the genes that were non-responsive to the decoy, might have a greater affinity for LXRα than the renin CNRE sequence used in the decoy molecules. Alternatively, although positive for the presence of the consensus CNRE sequence, these genes might be regulated by other transcriptional mechanisms described above such as the binding of cAMP-activated LXRα to a sequence distinct from the CNRE or the regulation of gene expression secondary to the induction of transcription factors by LXRα.

EMSA assays were performed to determine if LXRα was directly interacting with the identified cis-element CNRE sequences present in the genes. Since the majority of the CNRE elements present in the identified genes were able to effectively compete the previously described renin CNRE element in the majority of genes (9/11) for LXRα binding, this indicates that these genes are temporally regulated, in part, by the direct binding to LXRα to these cis-elements in As4.1 cells. Interestingly, the identified CNRE element(s) present in two genes (*RP11-492E24* and *Mrpl9*) were unable to compete for LXRα binding. One likely explanation could be that these particular genes are indirectly regulated by other genes which contain a functional CNRE element, and thus, directly regulated by LXRα. Other possible explanations could be that the sequences that flank these particular CNRE elements reduce the affinity to effectively compete the renin CNRE probe, or that the functional CNRE element lies outside of the queried region (50Kb). Whether this occurs under physiological conditions, with endogenous levels of LXRα remains to be determined.

The identification of a set of genes that are regulated by LXRα through the CNRE element suggests that this mechanism of cAMP-mediated induction may be a specific gene regulatory system when compared to the widespread effects of CRE-mediated induction. Indeed, LXRα has been reported to be a key mediator in the regulation of expression of genes that tend to maintain cellular or systemic physiological homeostasis (1). Moreover, these genes are regulated by LXRα through a specific DNA enhancer sequence (DR4/LXRE). Our data suggest LXRα as being a key mediator in the induction of other genes in mouse kidney cells though the CNRE element and indicate that modulation of transcription via CNRE is most likely a temporal and/or cell type specific mechanism of

cAMP induction when compared to CRE mediated induction. The human renin gene also contains a CNRE in addition to a CRE element which indicates the need for a more detailed regulatory system in response to cAMP. It will be interesting to determine how these newly identified LXRα/CNRE regulated genes play a role in the overall cellular physiology in kidney cells in response to cAMP and the teleological importance of gene modulation exerted by this specific mechanism.

In summary, in this communication we have utilized a genomics approach, by querying a 19,064-element mouse cDNA microarray, generated in our lab, for potential cAMP-activated LXRα regulated genes. Our data, taken together with previous reports, indicate that LXRα can regulate the expression of different sets of genes using multiple mechanisms (i.e. heterodimerization at the DR4 element, monomer at the CNRE) to regulate different physiological functions. LXRα not only regulates cholesterol metabolism and bile acid production, but also renin expression, a major mediator of blood pressure homeostasis and kidney function. These data would suggest that LXRα is an important transcriptional regulator of the cardiovascular system and cellular physiology.

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FIGURE LEGENDS

Fig. 1 Hierarchical cluster analysis of differentially expressed genes in mouse As4.1 JG cells. As described in the results, As4.1/GFP or As4.1/LXRα cells were treated with 1mM8-Br-cAMP or vehicle (PBS) for 1, 6, 12, or 24 hours. Profiling was performed using a custom 19,064 element array produced in-house. The data was filtered so as to include only genes (11,528) which demonstrate differential expression in at least one condition. The results indicate that LXRα/cAMP has a dramatic effect on the profiles of expression in As4.1 juxtaglomerular cells.

Fig. 2 Mouse renin (Ren-1d) Expression in As4.1 cells in presence of LXRα and cAMP**.** As4.1 cells that were stably transduced with a retrovirus expressing either a bicistronic GFP/LXRα construct or GFP, were treated with 1mM 8-Br-cAMP or Vehicle (PBS) for the times indicated. Total RNA was isolated and used as template for RT-PCR amplification using primers specific for mouse renin. Results by either agarose gel analysis (**A**), or average luminosity signal intensity (**B**) indicate that renin is induced, with a maximal peak, at 6 hours poststimulation in LXRα transduced As4.1 cells when compared to control cells (As4.1/GFP + Vehicle).

Fig. 3 Hierarchical cluster of genes that exhibit a renin expression profile. Hierarchical clustering (**A**) of genes that exhibit a highly similar (correlation ≥0.92) temporal expression profile to mouse renin (**B** and **C**) were clustered using a Pearson centered correlation coefficient distance metric (separation ratio= 0.9; minimum distance=0.001). The results indicate the existence of groups of genes that tend to cluster separately from the full list.

Fig. 4 Multiplex RT-PCR validation of genes that contain a CNRE element. Total RNA was isolated from As4.1/GFP and As4.1/LXRα, which were treated with vehicle or 8-Br-cAMP for 6 hours. Multiplex RT-PCR amplification was performed using gene-specific and 18s RNA primers. Results indicate that 15 out of 16 genes analyzed display the expected increase in mRNA abundance in cAMP treated As4.1/LXRα cells. No significant difference was observed with 18s RNA signal

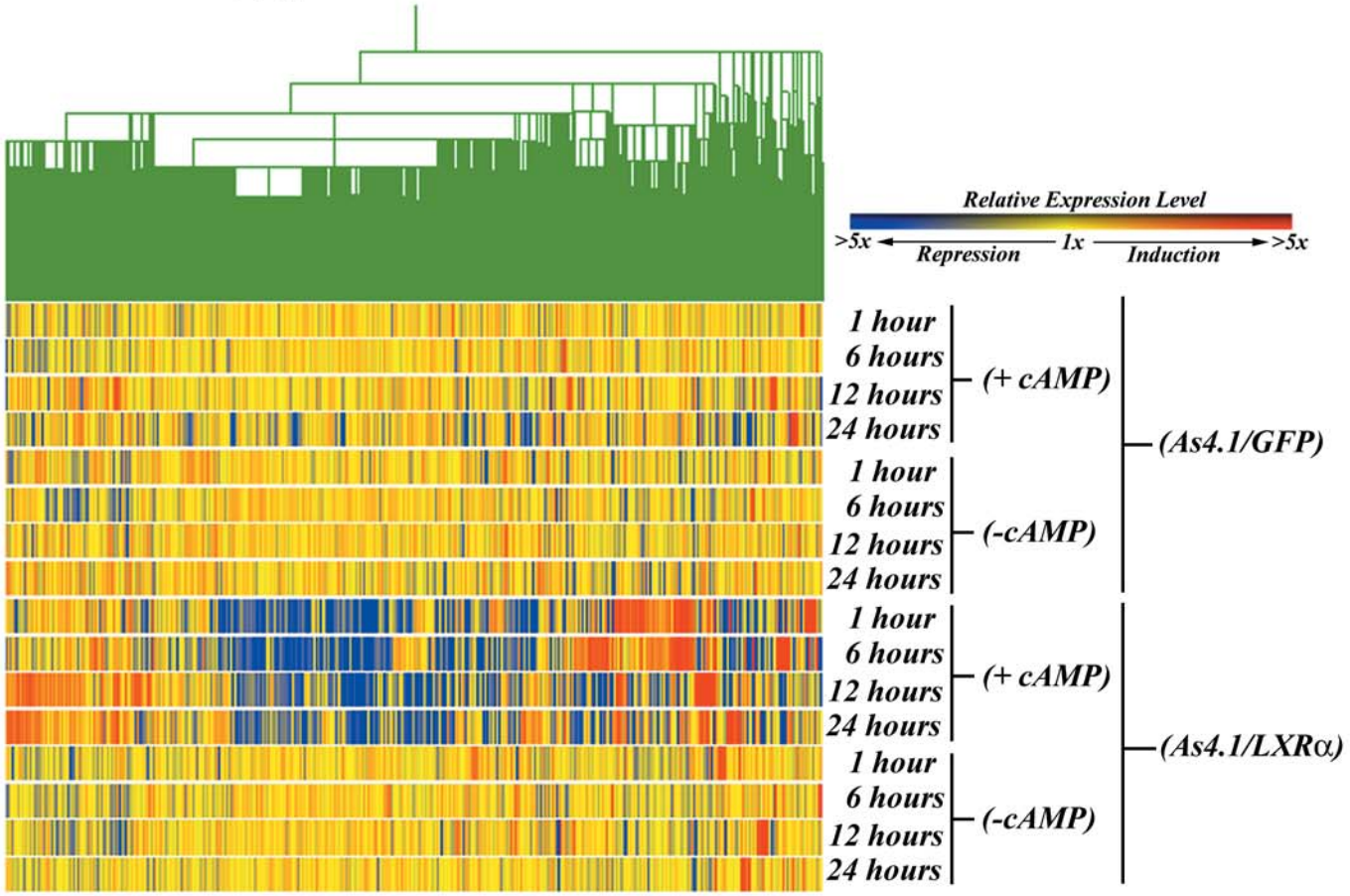
Fig. 5 Assessment of Decoy Transduction Efficiency in As4.1 Cells. Double-stranded FITC-labeled oligonucleotide (decoy), containing either CNRE, CRE, or scrambled DNA sequence, was transduced (40mM) into

As4.1 JG cells. After 48 hours, assessment of transduction efficiency was conducted using fluorescent and phase contrast microscopy. The number of positively transduced cells (fluorescent), transduced with CNRE, CRE, or scrambled DNA, was significantly higher than mock transduced cells (no-decoy). No significant difference was observed between cells transduced with CNRE, CRE, or scrambled decoy.

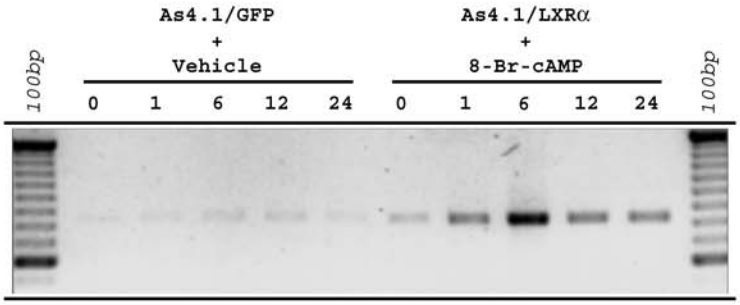
Fig. 6 Effects of decoy administration on LXRα regulated genes. As4.1/LXRα cells were transduced with the indicated double-stranded DNAs as described in the Methods section. Cells were then exposed to cAMP or vehicle for 6 hours and then harvested for RNA isolation. The isolated RNA was subjected to multiplex RT-PCR using primers for the indicated transcripts. **A**, The Ren-1d PCR product was greatly diminished in cells treated with the CNRE decoy but not in cells treated with the CRE decoy or with scrambled double-stranded DNA. **B**, 11 out of 16 transcripts were decreased following exposure of the cells with the CNRE decoy but not the CRE decoy or with scrambled double-stranded DNA.

Fig.7 Electrophoretic mobility shift assays of LXRα binding to novel CNRE enhancer elements present in identified genes. Nuclear extracts were prepared from cAMP stimulated As4.1/LXRα or As4.1/GFP cells and 10μg used to assess interaction of LXRα to novel CNRE elements by competition or direct binding assays. **A**, Effects of 100-fold molar excess unlabeled probe, containing the cis-element CNRE sequence with flanking sequences, to compete with a ^{32}P-labeled probe containing the CNRE sequence present in mouse renin promoter. The CNRE element(s) present in 9 of the 16 identified genes were able to effectively compete for binding (noted with an asterisk). **B**, Nuclear extracts were incubated with ^{32}P-labeled probe containing the CNRE element present in either mouse *ren-1^d*, or the newly identified *psx-1*, or *KIAA0877* genes. A strong shift is observed using the psx-1 probe which is comparable to the renin CNRE probe. No gelshift was observed from the *KIA0877* probe. Moreover, abolishment of gelshift was observed when using an anti-LXRα antibody when compared to controls (IgG or no antibody).

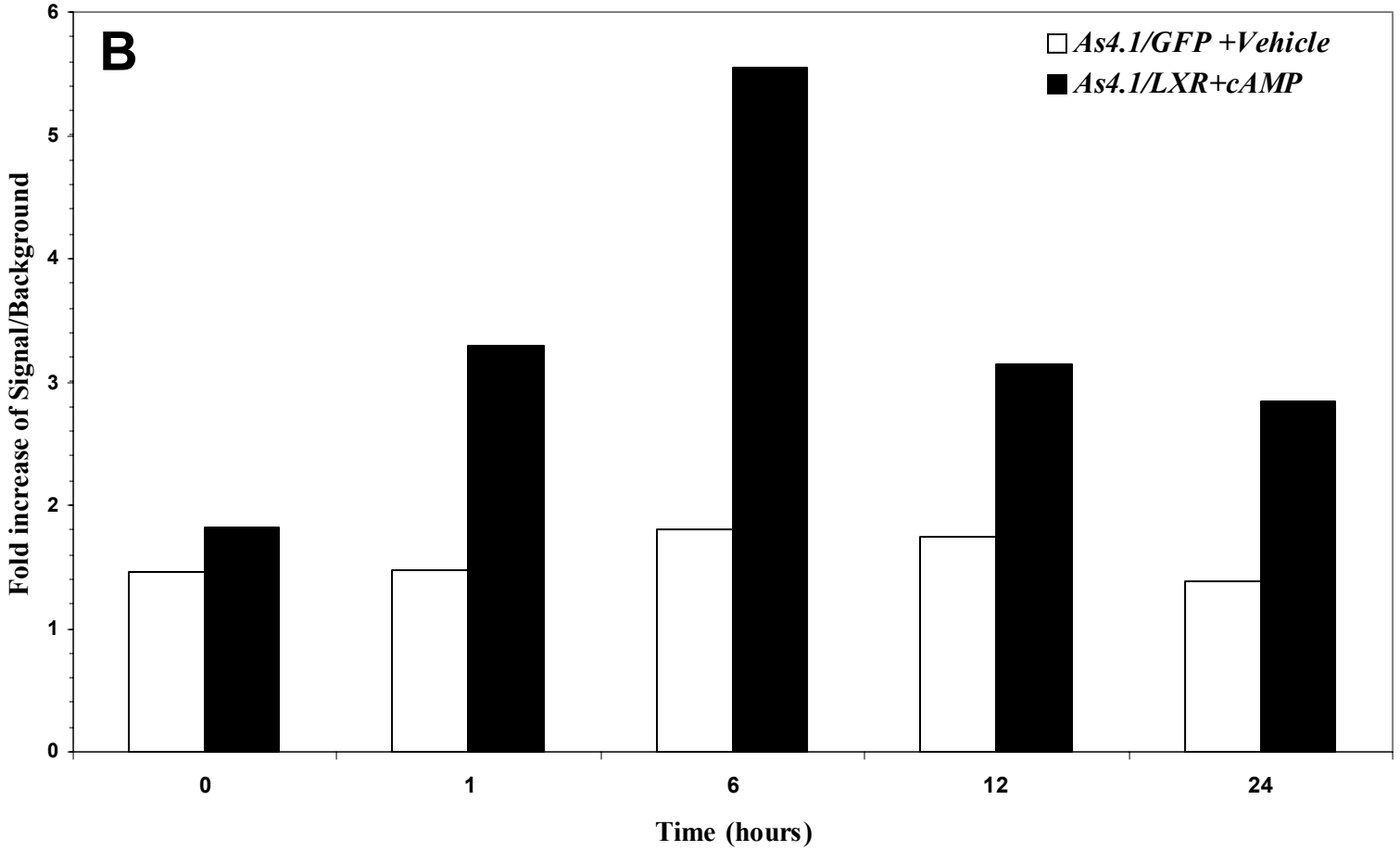
L. Anderson et al._Fig.1 **TOP**

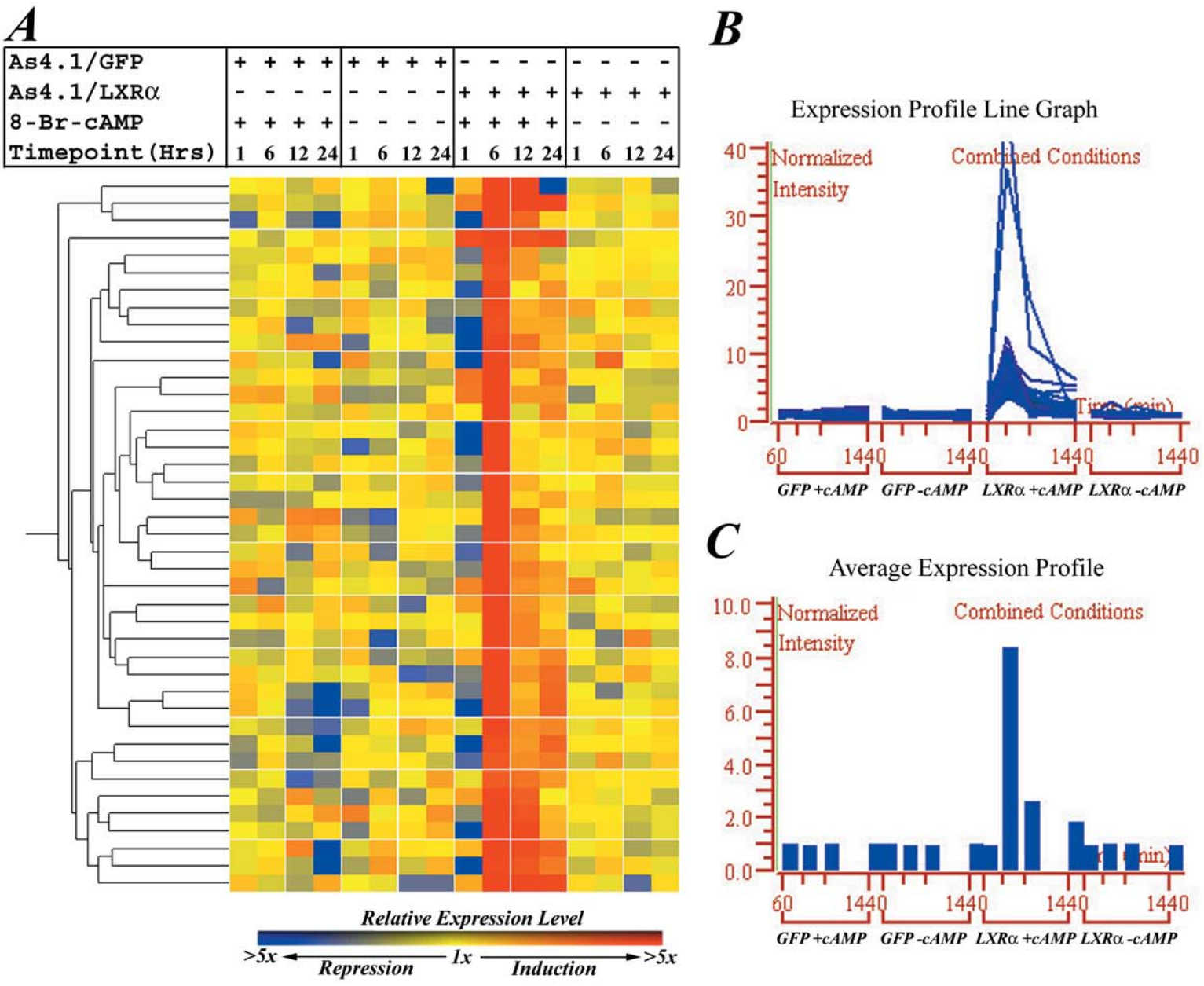


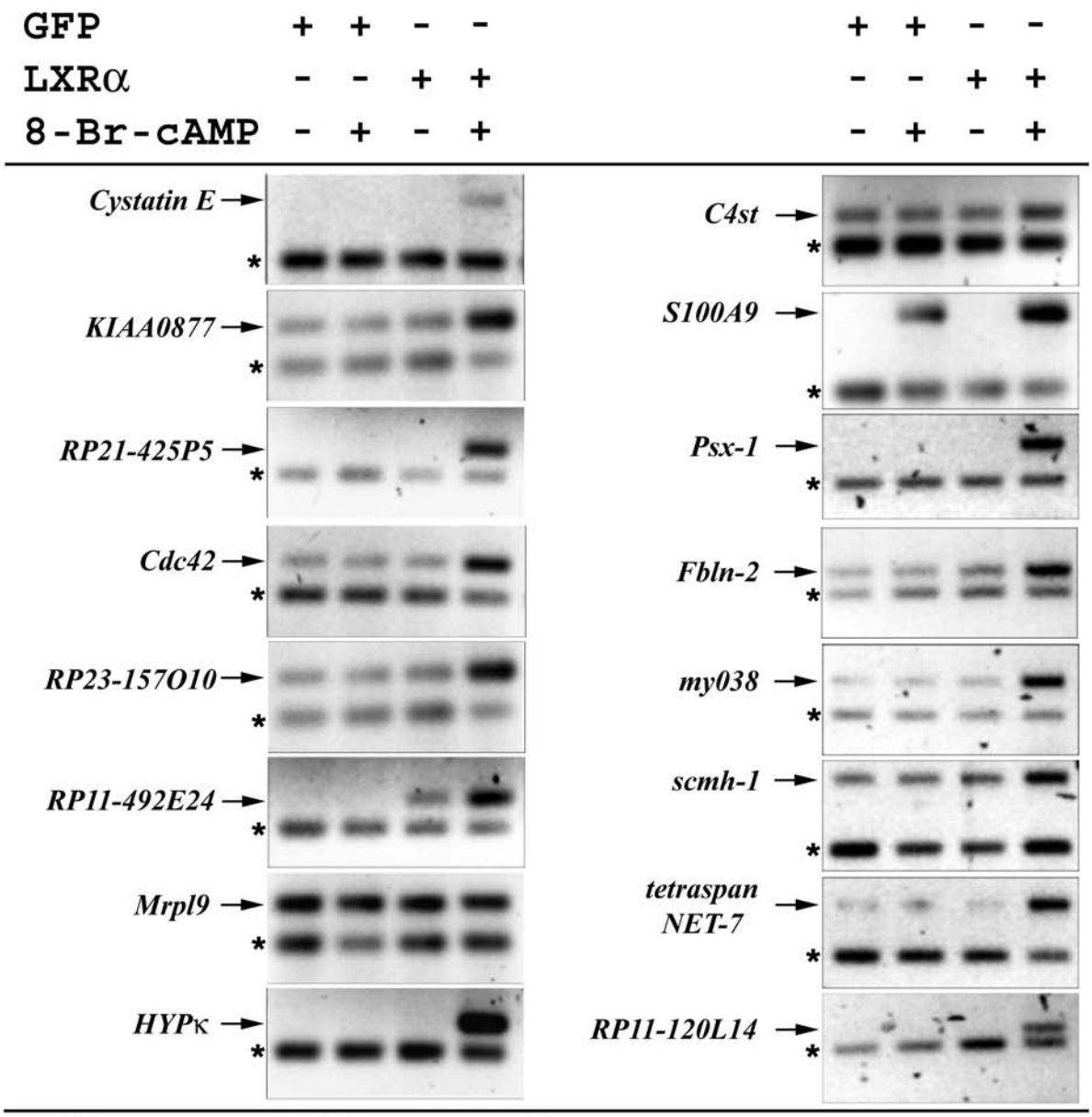
L.Anderson et.al._Fig2A



Renin product=823bp

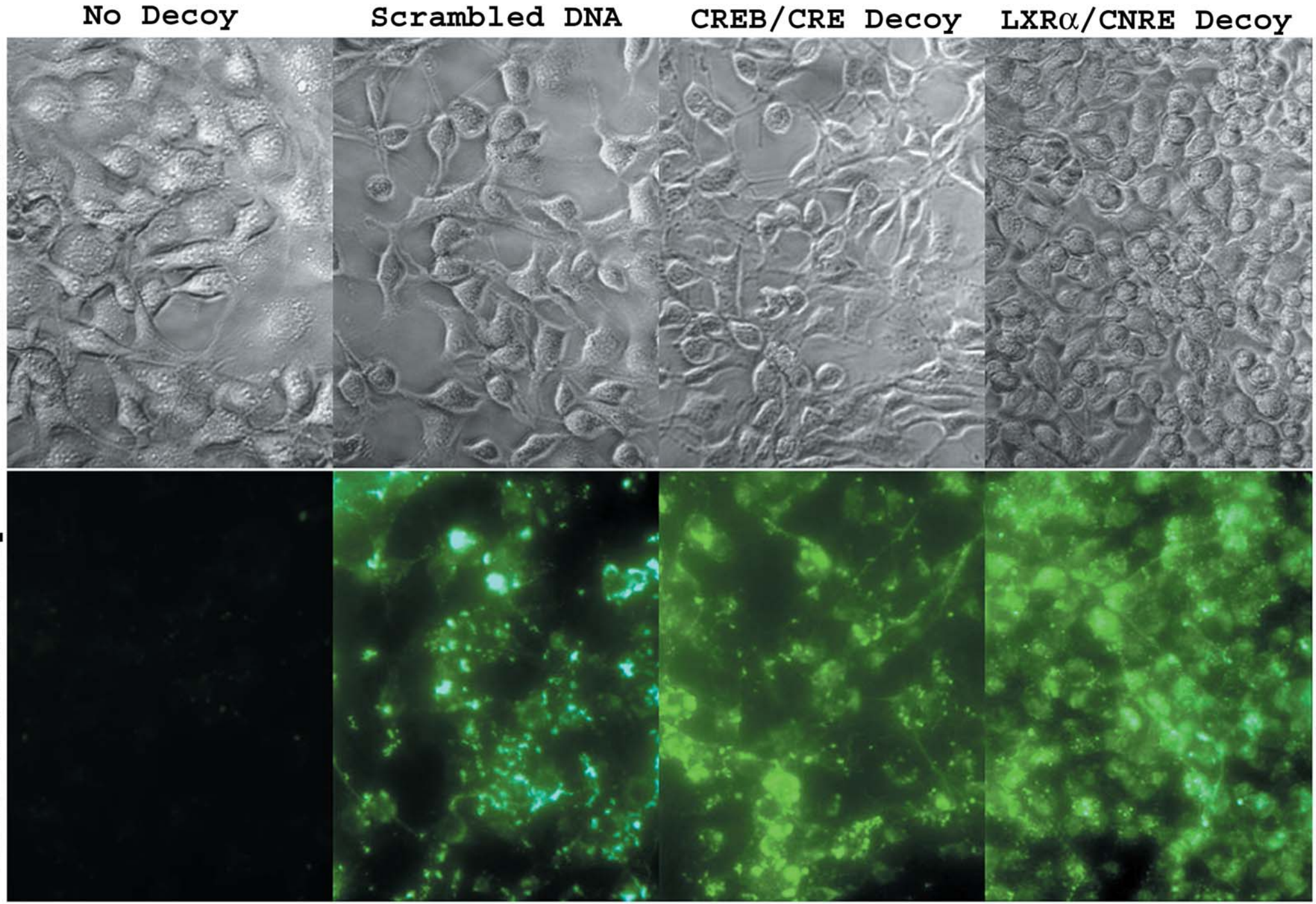

B
As4.1/GFP +Vehicle
As4.1/LXR+cAMP
Fold increase of Signal/Background
0
1
2
3
4
5
6
0
1
6
12
24
Time (hours)


A
As4.1/GFP + + + + + + + + - - - - - - - -
As4.1/LXRα - - - - - - - - + + + + + + + +
8-Br-cAMP + + + + - - - - + + + + - - - -
Timepoint(Hrs) 1 6 12 24 1 6 12 24 1 6 12 24 1 6 12 24
Relative Expression Level
>5x Repression 1x Induction >5x
B
Expression Profile Line Graph
Normalized Intensity
Combined Conditions
40
30
20
10
0
60 1440 1440 1440 1440
GFP +cAMP GFP -cAMP LXRα +cAMP LXRα -cAMP
C
Average Expression Profile
Normalized Intensity
Combined Conditions
10.0
8.0
6.0
4.0
2.0
0.0
60 1440 1440 1440 1440
GFP +cAMP GFP -cAMP LXRα +cAMP LXRα -cAMP



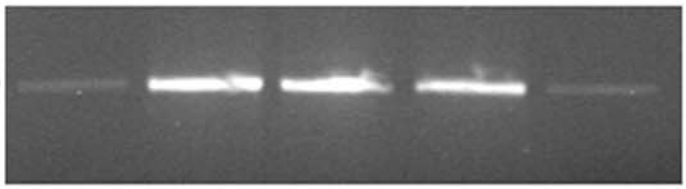
(18S RNA Multiplex Control)

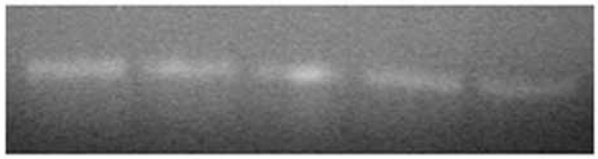
TOP


No Decoy
Scrambled DNA
CREB/CRE Decoy
LXRα/CNRE Decoy
Phase Contrast
FITC-Labeled Decoy

A

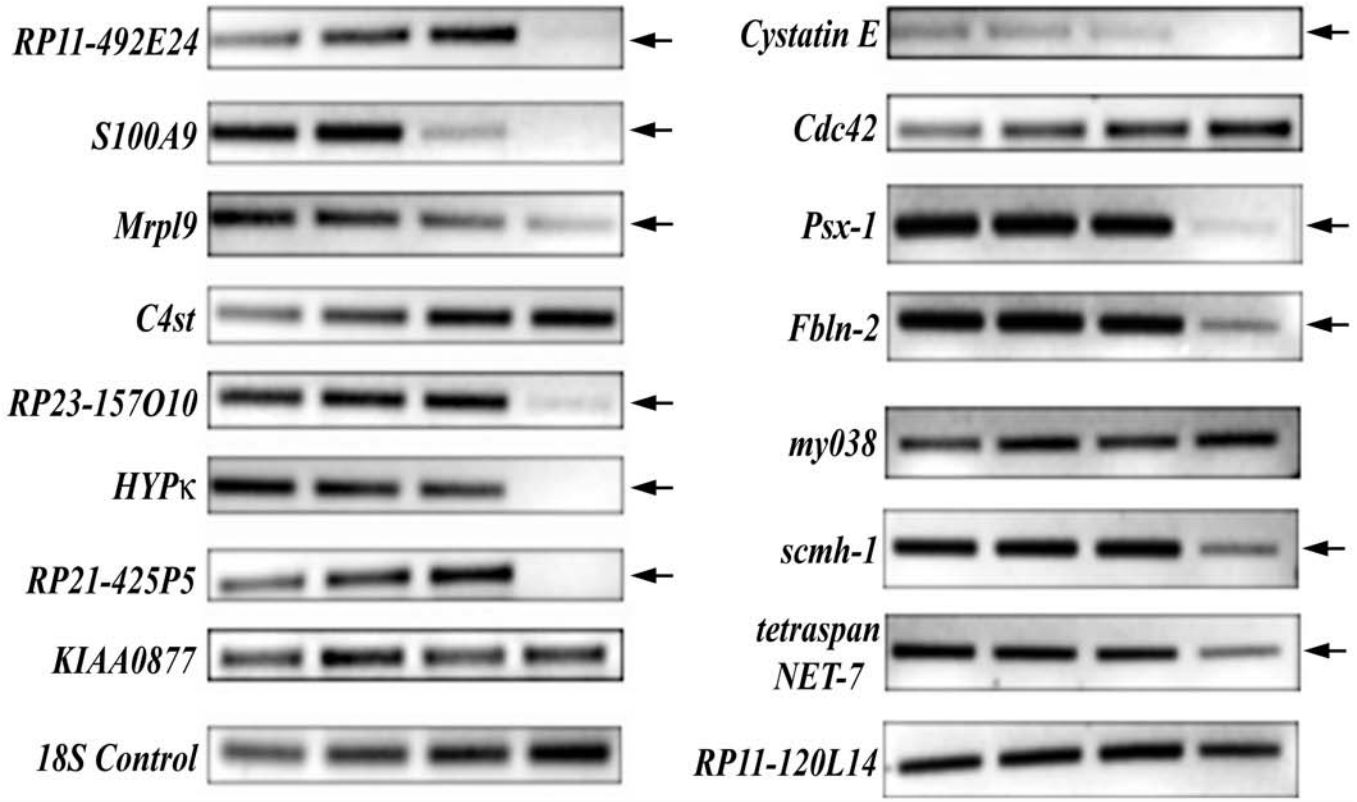
8-Br-cAMP	-	+	+	+	+
No Decoy	+	+	-	-	-
Scrambled DNA	-	-	+	-	-
CREB/CRE Decoy	-	-	-	+	-
LXRa/CNRE Decoy	-	-	-	-	+

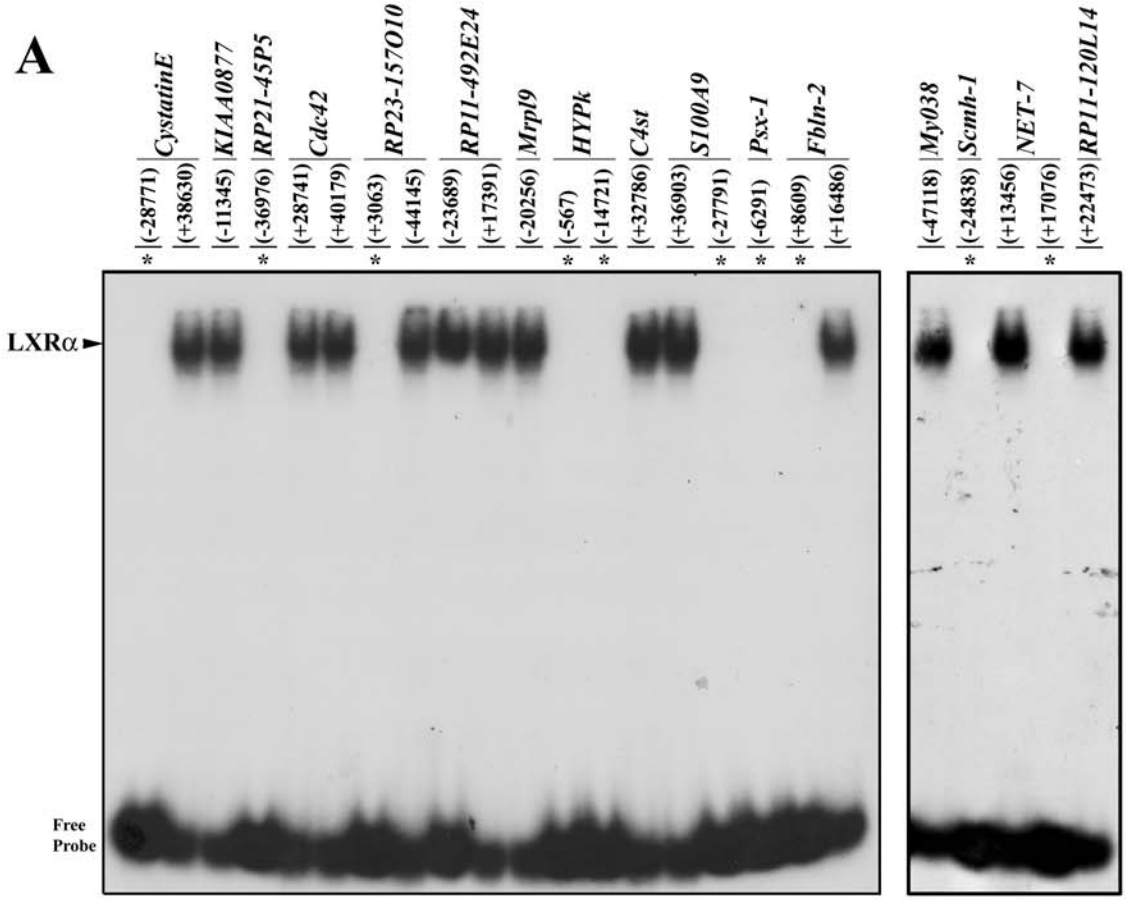




B

Scrambled DNA	-	+	-	-		-	+	-	-
CREB/CRE Decoy	-	-	+	-		-	-	+	-
LXRα/CNRE Decoy	-	-	-	+		-	-	-	+




A
CystatinE (-28771) (+38630)
KIAA0877 (-11345) (-36976)
RP21-45P5 (+28741) (+40179)
Cdc42 (+3063) (-44145)
RP23-157O10 (-23689) (+17391)
RP11-492E24 (-20256)
Mrpl9 (-567) (-14721)
HYPk (+32786)
C4st (+36903) (-27791)
S100A9 (-6291) (+8609)
Psx-1 (+16486)
Fbln-2 (-47118) (-24838)
My038 (+13456) (+17076)
Scmh-1 (+22473)
NET-7
RP11-120L14
LXRα
Free Probe

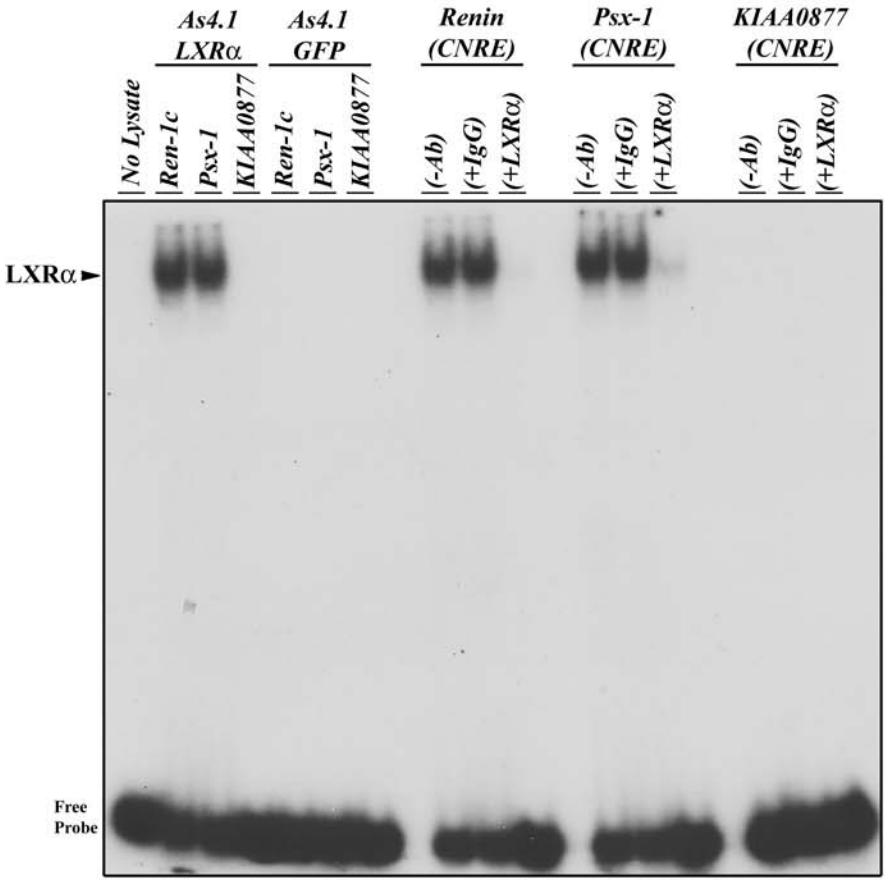

B
As4.1 LXRα
As4.1 GFP
Renin (CNRE)
Psx-1 (CNRE)
KIAA0877 (CNRE)
No Lysate
Ren-1c
Psx-1
KIAA0877
Ren-1c
Psx-1
KIAA0877
(-Ab)
(+IgG)
(+LXRα)
(-Ab)
(+IgG)
(+LXRα)
(-Ab)
(+IgG)
(+LXRα)
LXRα
Free Probe

Table 1: Expressed Sequence Tag (EST) Distribution of Spotted Clones on Mouse Array

In House Clones	
Number of ESTs Matched to Known Genes	***2,426***
Number of ESTs Matched to other ESTs in Unigene Database	***460***
Number of ESTs Unmatched to other Libraries (Novel)	***146***
NIA 15k Set	
Number of ESTs Matched to Known Genes	***4,020***
Number of ESTs Matched to other ESTs in Unigene Database	***5,530***
Number of ESTs Unmatched to other Libraries (Novel)	***5,714***
Total number of spotted ESTs	***18,296***
Number of Bacterial Controls	***768***
Total Number of clones spotted on Mouse cDNA Array	***19,064***

Sequences from the in house clones or from the NIA 15k clone set were blasted against the Unigene Database (Build#88) to generate a distribution based on match to known genes or other ESTs.

Table 2: Expressed Genes that Exhibit a Temporal Expression Profile Similar to Mouse Renin

MouseArray-ID	Accession Number	Correlation Coefficient	Clone Description	Fold Increase at 6hrs	T-test *p*-Value	Gene Function
Gene-7570	BG082494	0.982	Homo sapiens mRNA for Tom22, complete cds	9.621	4.99E-02	Matrix/Structural
Gene14280	N28198	0.971	Human cystatin E mRNA	6.542	1.19E-03	Signal Transduction
Gene11349	BG085590	0.967	Mus musculus ubiquitin fusion degradation 1 like (Ufd1l), mRNA	5.785	1.60E-02	Protein Synthesis
Gene18778	BG088787	0.963	Homo sapiens mRNA for KIAA0877 protein, partial cds	7.944	6.68E-03	Undetermined
Gene11822	BG073218	0.963	Mus musculus placenta specific homeobox 1 (Psx1), mRNA	6.238	9.08E-03	Transcription/Chromatin
Gene16191	BG074242	0.962	Mus musculus similar to hypothetical protein FLJ22170	6.947	8.86E-03	Undetermined
Gene-5894	C88185	0.960	Homo sapiens mRNA for KIAA1335 protein, partial cds	7.059	7.38E-03	Undetermined
Gene14043	R75296	0.958	Cavia porcellus GEC-1 (gec-1) mRNA, 3'UTR	7.47	6.21E-03	Undetermined
Gene-6022	BG068093	0.957	Mus musculus similar to Ank repeat containing protein (Pfam)	6.071	7.29E-03	Matrix/Structural
Gene14112	MDB1442R	0.957	Homo sapiens chromodomain helicase DNA binding protein 4 (CHD4)	5.577	1.19E-02	Transcription/Chromatin
Gene-3122	BG065549	0.954	Mus domesticus strain MilP mitocondrion genome, complete sequence	7.096	9.03E-03	Undetermined
Gene18766	BG076251	0.953	Homo sapiens mRNA; cDNA DKFZp434A0312 (from clone DKFZp434A0312)	6.013	9.95E-03	Undetermined
Gene11449	AW547303	0.953	Mus musculus ATP-binding cassette transporter sub-family A member 7 (Abca7)	10.514	7.08E-03	Matrix/Structural
Gene-8648	BG070409	0.948	Mus musculus lens intrinsic membrane protein 2 (Lim2)	9.464	6.25E-03	Matrix/Structural
Gene-5776	BG067871	0.946	Mus musculus cyclin I (Ccni) gene	10.906	5.71E-02	Cell Growth and Maintanence
Gene11831	BG085966	0.945	Mus musculus fibulin 2 (Fbln2), mRNA	6.948	1.15E-02	Matrix/Structural
Gene14452	AF108357	0.941	Mus musculus c-myc binding protein MM-1 (Mm-1) mRNA	8.975	5.73E-02	Undetermined
Gene-1063	BG077186	0.939	Mus musculus heat shock 70 protein (Hsc70) gene, complete cds	6.631	7.86E-03	Heat Shock/Stress
Gene11444	BG085672	0.939	Homo sapiens full length insert cDNA clone ZC48G12	6.051	8.21E-03	Undetermined
Gene-3626	BG065995	0.938	Mus musculus similar to hypothetical brain protein my038	6.149	1.12E-02	Undetermined
Gene10507	BG072071	0.938	Mus musculus cell division cycle 42 homolog (S. cerevisiae) (Cdc42), mRNA	7.19	9.89E-03	Cell Growth and Maintanence
Gene-4432	BG066540	0.935	Homo sapiens cDNA: FLJ21033 fis, clone CAE09040	7.488	7.11E-03	Undetermined
Gene11509	BG085732	0.934	Mus musculus CDC10 gene, promoter, exon 1 and joined CDS	4.596	1.80E-02	Undetermined
Gene-8934	BG070686	0.934	Mus musculus P450 (cytochrome) oxidoreductase (Por), mRNA	37.109	1.78E-03	Matrix/Structural
Gene13982	AK011163	0.933	Mouse DNA sequence from clone RP23-157O10 on chromosome 11	8.242	8.49E-03	Undetermined
Gene10858	BG072396	0.927	Homo sapiens chromosome 2 clone RP11-492E24	3.445	2.14E-02	Undetermined
Gene11149	BG072628	0.926	Mus musculus scmh1 mRNA for sex comb on midleg homolog protein, complete cds	3.858	2.07E-02	Transcription/Chromatin
Gene17364	BG075147	0.926	Homo sapiens hypothetical protein (CL25022), mRNA	4.742	1.47E-02	Undetermined
Gene11274	BG085528	0.926	Homo sapiens transmembrane 4 superfamily member (tetraspan NET-7) (NET-7), mRNA	8.715	6.69E-03	Matrix/Structural
Gene-3020	BG078740	0.925	Mus musculus mitochondrial ribosomal protein L9 (Mrpl9)	9.136	6.49E-03	Undetermined
Gene11347	BG072801	0.924	Mus musculus S100A9 gene for S100A9 protein exons 1-3	4.417	1.91E-02	Signal Transduction
Gene12909	AA170276	0.924	EST(ms82e12.r1 Soares mouse 3NbMS clone 618094 5')	5.916	1.09E-02	Undetermined
Gene15540	BG086415	0.923	Homo sapiens Huntingtin interacting protein K (HYPK), mRNA	11.084	9.33E-03	Matrix/Structural
Gene14446	L02210	0.923	Mus musculus tyrosine kinase-related protein mRNA	12.632	3.51E-03	Signal Transduction
Gene14575	NM007663	0.923	Mus musculus cadherin 16 (Cdh16), mRNA	3.931	9.50E-02	Matrix/Structural
Gene-3506	C77298	0.922	Human DNA sequence from clone RP11-120L14 on chromosome 13	6.881	9.68E-03	Undetermined
Gene-3443	BG079106	0.921	Mus musculus Y-box binding protein (oxyR) mRNA, partial cds	5.145	1.03E-02	Transcription/Chromatin
Gene-7564	BG069383	0.921	Homo sapiens chromosome 16 clone RP11-256I9	10.245	5.73E-03	Undetermined
Gene18927	BG088903	0.921	Homo sapiens fasciculation and elongation protein zeta 1 (zygin I) (FEZ1), mRNA	51.692	1.33E-03	Signal Transduction
Gene-839	BG077002	0.920	Homo sapiens mRNA for KIAA1398 protein, partial cds	6.623	1.10E-02	Undetermined
Gene-1812	BG064375	0.920	Mus musculus mRNA for chondroItin 4-sulfotransferase, complete cds	4.864	1.37E-02	Matrix/Structural

The entire list of expressed genes (11,529) were queried using the temporal expression profile of mouse renin(Ren-1d) and resulted in the identification of 41 genes which display a profile similar to that of renin (correlation ^{3}0.92). Gene function annotation is derived from GeneSpring and is based on Gene Ontology (GO) consortium classification.

Table 3: List of Genes with Celera™ Database Identified CNRE Element

Mouse Array-ID	Accession Number	Celera™ Contig No.	Clone Description	Distance of CNRE Element	Sequence Oreintation	CNRE Sequence	Percent Identity to Renin CNRE
Gene14280	*N28198*	*GA_x5J8B7W38UJ*	*Human cystatin E mRNA*	*-28771*	*Reverse*	*TTGGTCCCAGAGG*	*84.6*
				38630	*Reverse*	*TACTTCTCACAGG*	*76.9*
Gene18778	*BG088787*	*GA_x5J8B7W6RL5*	*Homo sapiens mRNA for KIAA0877 protein, partial cds*	*-11345*	*Reverse*	*TGGTTCCCACAGG*	*84.6*
Gene-8648	*BG070409*	*GA_x5J8B7W546B*	*Mus musculus, chromosome 16q, clone:RP21-425P5*	*-36976*	*Reverse*	*TCATTCCCAGAGG*	*69.2*
Gene10507	*BG072071*	*GA_x5J8B7W7N76*	*Mus musculus cell division cycle 42 homolog (S. cerevisiae) (Cdc42)*	*28741*	*Reverse*	*TGGGTCCCACAGG*	*84.6*
				40179	*Reverse*	*TGTGTCTCACAGG*	*84.6*
Gene13982	*AK011163*	*GA_x5J8B7W82RK*	*Mouse DNA sequence from clone RP23-157O10 on chromosome 11*	*3063*	*Reverse*	*TTGGTCCCAGAGG*	*84.6*
				-44145	*Forward*	*TCTGTCCCACAGG*	*76.9*
Gene10858	*BG072396*	*GA_x5J8B7W6DHQ*	*Homo sapiens chromosome 2 clone RP11-492E24*	*-23689*	*Reverse*	*TCTTTCCCAGAGG*	*61.5*
				17391	*Reverse*	*TTTTTCCCACAGG*	*76.9*
Gene-3020	*BG078740*	*GA_x5J8B7W33D5*	*Mus musculus mitochondrial ribosomal protein L9 (Mrpl9)*	*-20256*	*Reverse*	*TGTTTCTCACAGG*	*76.9*
Gene15540	*BG086415*	*GA_x5J8B7W62CR*	*Homo sapiens Huntingtin interacting protein K (HYPK), mRNA*	*-567*	*Forward*	*TTTGTCCCAGAGG*	*76.9*
				-14721	*Forward*	*TCAGTCTCACAGG*	*84.6*
Gene-1812	*BG064375*	*GA_x5J8B7W8864*	*Mus musculus mRNA for chondroItin 4-sulfotransferase, complete cds*	*32786*	*Reverse*	*TGTTTCTCACAGG*	*76.9*
Gene11347	*BG072801*	*GA_x5J8B7W7N76*	*Mus musculus S100A9 gene for S100A9 protein exons 1-3*	*36903*	*Forward*	*TCCTTCTCAGAGG*	*69.2*
				-27791	*Forward*	*TTCTTCTCAGAGG*	*76.9*
Gene11822	*BG073218*	*GA_x5J8B7W4MNF*	*Mus musculus placenta specific homeobox 1 (Psx1), mRNA*	*-6291*	*Reverse*	*TGCTTCTCACAGG*	*76.9*
Gene11831	*BG085966*	*GA_x5J8B7W7FK3*	*Mus musculus fibulin 2 (Fbln2), mRNA*	*8609*	*Forward*	*TGTTTCTCACAGG*	*76.9*
				16486	*Reverse*	*TCTGTCTCAGAGG*	*76.9*
Gene-3626	*BG065995*	*GA_x5J8B7W4L8W*	*Mus musculus similar to hypothetical brain protein my038*	*-47118*	*Reverse*	*TGCTTCCCACAGG*	*69.2*
Gene11149	*BG072628*	*GA_x5J8B7W3HRA*	*Mus musculus scmh1 mRNA for sex comb on midleg homolog protein*	*-24838*	*Reverse*	*TCTGTCCCAGAGG*	*69.2*
Gene11274	*BG085528*	*GA_x5J8B7W4K2N*	*Homo sapiens transmembrane 4 superfamily member (tetraspan NET-7)*	*13456*	*Forward*	*TAAGTCTCAGAGG*	*76.9*
				17076	*Forward*	*TAATTCTCAGAGG*	*69.2*
Gene-3506	*C77298*	*GA_x5J8B7W3WK4*	*Human DNA sequence from clone RP11-120L14 on chromosome 13*	*22473*	*Forward*	*TGAGTCTCACAGG*	*84.6*

Using the Celera database, fifty kilobases (50Kb) of flanking sequence was queried for the presence of the consensus CNRE element. Since most the ESTs to be queried are not matched to known genes, a 50Kb distance was utilized to ensure the probability of analyzing a promoter region. The upstream region of the 41 EST sequences were used as an 'anchor' and resulted in 10 genes that contained a consensus CNRE within a 50Kb upstream region. The total number of genes could further be divided into groups that contained CNRE elements within a short (2), moderate (4) and long distance (4) from the anchor sequence. Comparison of the CNRE sequences to mouse Ren-1d CNRE resulted in an identity range of 61.5-84.6%.