CRTR-1, a Developmentally Regulated Transcriptional Repressor Related to the CP2 Family of Transcription Factors*

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CP2-related proteins comprise a family of DNA-binding transcription factors that are generally activators of transcription and expressed ubiquitously. We reported a differential display polymerase chain reaction fragment, \textit{Psc}2, which was expressed in a regulated fashion in mouse pluripotent cells \textit{in vitro} and \textit{in vivo}. Here, we report further characterization of the \textit{Psc}2 cDNA and function. The \textit{Psc}2 cDNA contained an open reading frame homologous to CP2 family proteins. Regions implicated in DNA binding and oligomeric complex formation, but not transcription activation, were conserved. \textit{Psc}2 expression \textit{in vivo} during embryogenesis and in the adult mouse demonstrated tight spatial and temporal regulation, with the highest levels of expression in the epithelial lining of distal convoluted tubules in embryonic and adult kidneys. Functional analysis demonstrated that \textit{Psc}2 repressed transcription 2.5–15-fold when bound to a heterologous promoter in \textit{ES}, 293T, and \textit{COS}-1 cells. The N-terminal 52 amino acids of \textit{Psc}2 were shown to be necessary and sufficient for this activity and did not share obvious homology with reported repressor motifs. These results represent the first report of a CP2 family member that is expressed in a developmentally regulated fashion \textit{in vivo} and that acts as a direct repressor of transcription. Accordingly, the protein has been named \textit{CP2-Related Transcriptional Repressor}-1 (CRTR-1).

The mouse transcription factor CP2 was identified as an activator of the mouse \textit{α}-globin gene, which binds a promoter element overlapping the CCAAT box (1–3). CP2 is the founding member of a group of highly conserved proteins identified in mice, humans, and chickens referred to as the CP2 family of transcription factors (4). Human cDNAs encoding multiple CP2-related proteins have been identified. These include human CP2 (2, 5) (also referred to as LSF and LBP-1c (6–8)), LBP-1d (8) (also known as LSF-ID (7)), an alternatively spliced form of LBP-1c (8), LBP-1a (8), LBP-1b (8) (an alternatively spliced form of LBP-1a (8)), and LBP-9 (9). The mouse protein NF2d9 shows 94% identity to LBP-1a and is recognized as the homologue (4), and a chicken CP2 homologue has also been reported (10).

Consistent with the ability of mouse CP2 to activate transcription, human CP2/LBP-1c has been shown to activate transcription from an SV40 promoter (6) and from cellular promoters such as those directing expression of the serum amyloid A3 gene (11). The ability to activate transcription is conserved among other family members; LBP-1b activates transcription from the –155/–131 region of the human \textit{P450scc} promoter (9), LBP-1a activates transcription from a human immunodeficiency virus, type I promoter (8), and chicken CP2 activates transcription from the \textit{α}-crystallin gene promoter (10).

Members of the CP2 family of transcription factors bind a consensus DNA sequence consisting of a direct bipartite repeat sequence, CNRG-N$_X$-CNRG (3, 10). Binding sites for this family of proteins have been described in the viral and cellular promoters described above; binding sites for CP2/LBP-1c have been described in the \textit{γ}-fibrinogen (12, 3), synthase kinase-3β (13), and \textit{γ}-globin (5) promoters; binding sites for \textit{NF2d9} have been described in the Cyp 2d-9 (steroid 16α-hydroxylase) promoter (4); and binding sites for LBP-9 have been described in the human \textit{P450scc} promoter (9). Amino acids 63–270 of CP2 share sequence similarity with the region required for DNA binding in the \textit{Drosophila melanogaster} transcription factor \textit{grainyhead} (\textit{grh}) (2). This region is highly conserved within other CP2 family members and appears to be important for DNA binding because LBP-1d, which is translated from an alternatively spliced form of CP2/LBP-1c and lacks amino acids 189–239, is unable to bind the LBP-1c DNA binding sequence (7, 8). N- and C-terminal truncation studies have defined the minimum DNA binding region of LBP-1c between amino acids 65 and 383 (14).

Human CP2 has been reported to bind DNA as a dimer (5, 7, 15), although other reports have shown that LBP-1c (14) and chicken CP2 (10) bind DNA as tetramers. Truncation studies have localized a region of LBP-1c required for oligomerization to amino acids 266–403 (14). Formation of hetero-multimers between CP2 family members LBP-1a, b, and c has also been reported (8). CP2 family members can form complexes with nonrelated cellular proteins. For example, LBP-1c interacts with YY1 on the human immunodeficiency virus, type I promoter (16), an unidentified protein (40–45 kDa) forming the stage selector protein complex that binds to the \textit{γ}-globin promoter (5) and a neuron-specific protein FE65 (17). Protein sequences required for hetero-oligomerization have not been defined.

Mammalian members of the CP2 family are generally expressed ubiquitously (4, 5, 10, 18). Whereas LBP-9 expression in cultured cell lines suggests some regulation of expression (9), the expression of this gene has not been mapped \textit{in vivo}. Using
differential display PCR analysis we identified three novel genes that exhibit regulated expression during pluripotent cell differentiation. Expression of these genes was temporally regulated during conversion of ES cells to EPL cells, an in vitro system that recapitulates conversion of inner cell mass to primitive ectoderm in vivo (19), and in the pluripotent cells of the pregastrulation mouse embryo. In this paper we report further analysis of one of these genes, denoted Psc2, which was expressed in pluripotent cells in vivo at 3.5 and 4.5 days post coitum (d.p.c.) and down-regulated around 4.75 d.p.c.. We demonstrate that the Psc2 cDNA encodes a novel mouse member of the CP2 family, which differs from the known members in two respects. Firstly, expression of this gene is tightly regulated in vivo in both temporal and spatial fashion, with the strongest expression detected in the epithelial lining of distal convoluted tubules (DCTs) in the embryonic and adult kidney. Secondly, the protein exhibits a novel transcriptional repression activity, localized in the N terminus of the protein, when tethered to a heterologous promoter. Accordingly, we have renamed the gene CP2-Related Transcriptional Repressor-1 (CRTR-1).

MATERIALS AND METHODS
cDNA Isolation and Sequencing—CRTR-1 cDNA clones were isolated from a λ ZAP II ES cDNA library derived from D3 ES cell RNA (Clontech). In library screening, clones were isolated by differential display previously (20) using random primed [α-32P]dATP-labeled (Geneworks Ltd., Adelaide, South Australia, Australia) DNA probes (Gigaprase kit, Geneworks Ltd.). Clones q1, 1.2, 6A, 8.2.1, and 8B were isolated by successive screening of the library with a 736-bp fragment isolated by EcoRI digestion of the cloned CRTR-1 differential display PCR fragment, a 132-bp fragment isolated by EcoRI/AatI digestion of cDNA clone q1, a 190-bp fragment isolated by EcoRINucI digestion of cDNA clone 1.2, a 384-bp fragment isolated by EcoRI digestion of cDNA clone 6A, and a 500-bp fragment isolated by EcoRI digestion of cDNA clone 8.2.1, respectively (see Fig. 1A). Third round duplicate positive plaques from each library screen were isolated, grown to high titer, and excised from each library screen were isolated, grown to high titer, and excised (see Fig. 1A). The amplified SR1 (5'-ATAAAGCTTGAGCTCA-

DNA Manipulations—A sequence encoding a complete CRTR-1 open reading frame was amplified by reverse transcriptase-PCR on D3 ES cell RNA using the SuperScript One-Step reverse transcriptase-PCR system (Life Technologies, Inc.) according to the manufacturer's instructions. Primers used for amplification were SR1 (5'-ATAAAGCTTGAGCTCA-

RNA Isolation, Riboprobe Synthesis, and Ribonuclease Protection Assays—Poly(A)+ cytoplasmic RNA was isolated from D3 ES and EPL (19) cells as described previously (29). Mouse embryos from 10.5 to 17.5 d.p.c. were isolated and homogenized, and total RNA was isolated using the guanidinium isothiocyanate method (30).

CRTR-1 antisense riboprobes for use in RNase protections were synthesized as described previously (31) by transcription of HincII linearized cDNA clone 1.2 (see Fig. 1A) with T3 RNA polymerase (Roche Molecular Biochemicals). Ribonuclease protection assays were performed on 10 μg of total RNA as previously described (31, 33) except that hybridizations were for 14 h at 45 °C. To reduce overexposure of the loading control, low specific activity mGAP probes were synthesized using 40 μCi of [α-32P]UTP in the reaction, whereas CRTR-1 probes were synthesized using 120 μCi of [α-32P]UTP. 37,000 counts/min of mGAP probe and 150,000 counts/min of all other probes were added to each hybridization. [α-32P]UTP was obtained from Geneworks Ltd.

Wholemount in Situ Hybridization and in Situ Hybridization—16.5-d.p.c. kidneys were isolated from BALB/c mouse embryos. Wholemount in situ hybridization was carried out as described elsewhere (32) except that 16.5-d.p.c. embryonic kidneys were prewashed for 20 min in radioimmunoprecipitation buffer three times before post-fixing, prehybridized, and washed at 65 °C. Probed embryonic kidneys were embedded in room temperature agarose for 10 min, heated in 100% methanol in isopropanol, and 2 × 15 min in Histo-Clear (National Diagnostics) before embedding in paraffin wax. 7-μm serial sections were cut using a Leica microtome, floated on water at 45 °C, placed onto silanized microscope slides, de-paraffinized in Histo-Clear, and rehydrated through a methanol series; then the sections were counterstained with methyl green and mounted with DePex and a coverslip. Sections were viewed using a Nikon Eclipse TE300 inverted microscope and photographed with Ektachrome 100 ASA slide film (Kodak).

Adult kidneys were dissected from BALB/c female mice and fixed as above. 7-μm serial sections were cut as described for wholemount in situ hybridization. Radiolabeled in situ hybridization was carried out as described elsewhere (34) with the following modifications. Sections were heated to 55 °C for 30 min before de-paraffinization in Histoclear. Sections were prehybridized at 52 °C for 1 h and washed twice in 2 × SSPE for 2 min prior to overnight hybridization with riboprobes. Post-hybridization washes were carried out prior to RNase digestion as follows: 50% formamide, 2 × SSPE, 0.1% SDS, 10 mm β-mercaptoethanol at 52 °C for 5 min; 50% formamide, 2 × SSPE, 10 mm β-mercaptoethanol at 52 °C for 5 min; 50% formamide, 2 × SSPE, 10 mm β-mercaptoethanol at 60 °C for 10 min; and twice in 2 × SSPE at room temperature for 5 min. Slides were air-dried and warmed to 37 °C.

Sections were counterstained with hematoxylin, mounted with DePex and a coverslip, viewed using light and dark field condensers on a Zeiss Axioplan microscope, and photographed with Ektachrome 160T ASA slide film (Kodak).

1 The abbreviations used are: PCR, polymerase chain reaction; ES, embryonic stem; EPL, early primitive ectoderm-like; d.p.c., days post coitum; DCT, distal convoluted tubule; bp, base pair(s); mGAP, mouse glyceraldehyde-3-phosphate dehydrogenase; SSPE, saline/sodium phosphate/EDTA; DBD, DNA binding domain; TK, thymidine kinase.

2 T. Pelton, S. Sharma, T. Schulz, J. Rathjen, and P. Rathjen, submitted for publication.
**RESULTS**

**CRTR-1 cDNA Isolation and Sequence**—Based on the known expression in pluripotent cells in vitro and in vivo, CRTR-1 cDNA clones were isolated from a D3 ES cell ZAP II cDNA library (CLONTECH Inc; (20)) using successively more 5’ CRTR-1-specific probes (Fig. 1A). Clones were confirmed to be CRTR-1-specific using Southern analysis (data not shown), sequence data, and expression analysis (Fig. 1B and data not shown). Ribonuclease protection using riboprobes generated from the CRTR-1 cDNA clone 1.2 demonstrated rapid down-regulation of CRTR-1 expression upon differentiation of ES cells to EPL cells (Fig. 1, B and C), consistent with the pattern described previously for the differential display PCR product using Northern blot and in situ hybridization.²

Both strands of CRTR-1 cDNA fragments were sequenced to generate the CRTR-1 cDNA sequence (GenBank™ accession number AF311309). The 9405-bp cDNA contained a poly(A) tail 196 bp downstream of a consensus polyadenylation signal (AATAAA), consistent with the typical positioning of the polyadenylation signal 10–30 bp upstream of the poly(A) tail (39). A 1446-bp open reading frame extended from nucleotide 92 to nucleotide 1537 and was followed by a long 3’ untranslated region of 7868 bp. The CRTR-1 protein predicted from this compiled sequence is 481 amino acids long, with a predicted molecular mass of 54,702 daltons. No significant reading frames were identified.

**CRTR-1 Sequence Analysis**—Comparison of the predicted CRTR-1 amino acid sequence with entries in protein sequence databases revealed considerable similarity to a group of proteins related to the mouse transcription factor CP2 (2) (Table I). Fig. 2 shows a multiple sequence alignment of reported CP2 family members with CRTR-1. Included in Table I and the multiple sequence alignment are the mouse family members CP2 (2) and NF2d9 (4); human family members LBP-1a, LBP-1b, LBP-1c, LBP-1d, and LBP-9 (9); and the DNA binding domain of the D. melanogaster protein GRH (40).

Conservation between CRTR-1 and the other mammalian proteins was extensive (Fig. 2) and extended across the CRTR-1 sequence, with the exception of amino acids 1–47 and 381–401, which were conserved only with LBP-9. Furthermore, the 51-amino acid deletion at position 189 specific to LBP-1d and the 37-amino acid insertion (amino acids 274–312) specific to LBP-1b were not found in CRTR-1. Similarity to GRH was confined to amino acids 632–865, shown to be sufficient for DNA binding to elements in the Dopa decarboxylase (Ddc) promoter (41). The failure of proteins containing deletions within this region (LBP-1b, LBP-1c, and the 37-amino acid insertion (amino acids 274–312) specific to LBP-1b) were not found in CRTR-1. Similarity to GRH was confined to amino acids 632–865, shown to be sufficient for DNA binding to elements in the Dopa decarboxylase (Ddc) promoter (41). The failure of proteins containing deletions within this region (LBP-1b, LBP-1c, LBP-1d, and LBP-9 (9); and the DNA binding domain of the D. melanogaster protein GRH (40).
with the identification of this region as a DNA binding sequence that is conserved in the CRTR-1 protein.

Truncation studies have localized an oligomerization domain within LBP-1c to amino acids 266–403 (14). This region was well conserved within CRTR-1, suggesting a potential for formation of homo- and hetero-oligomeric protein complexes. Within the equivalent regions of CP2 (amino acids 398–425) and LBP-1c/LBP-1d are located a glutamine/proline repeat and a polglyutamine repeat, respectively, which have been predicted to form a transcriptional activation domain (2) but are not conserved in CRTR-1 or LBP-9.

CRTR-1 (9) is the CP2 family member that shows the greatest level of similarity to CRTR-1 (Table I; Fig. 2). Whereas there was considerable conservation of amino acid sequences between these proteins, similarity was restricted to the open reading frame and did not extend into the reported, incomplete 3’ untranslated region of LBP-9.

Regulated Expression of CRTR-1 during Mouse Development—Expression of CRTR-1 has been shown to be specifically regulated in pluripotent cell populations in vitro and in vivo. CRTR-1 expression during later mouse development was investigated by ribonuclease protection analysis using total embryonic RNA isolated from 10.5–17.5 d.p.c. embryos, tissue-specific total RNA samples isolated from 16.5–d.p.c. embryos, and tissue-specific total RNA samples isolated from adult mice. CRTR-1 expression was not detected in total RNA isolated from 12.5- and 13.5-d.p.c. embryos and was expressed at low levels in 10.5- and 11.5-d.p.c. embryos (Fig. 3A). CRTR-1 expression was highest between 14.5 and 17.5 d.p.c. Of the 16.5-d.p.c. embryonic tissues analyzed (Fig. 3B), CRTR-1 was not detected in 16.5-d.p.c. embryonic brain. Low levels of CRTR-1 expression were detected in 16.5-d.p.c. embryonic intestine, limb, lung, and skin, with highest expression in 16.5-d.p.c. embryonic kidney. Levels of CRTR-1 expression observed in 16.5-d.p.c. embryonic kidney were comparable with levels of expression observed in ES cells. Of the tissue-specific total RNA samples isolated from adult mice, CRTR-1 was not detected in brain, heart, liver, and spleen (Fig. 3C). CRTR-1 was expressed at low levels in lung, mesenteric lymph nodes, muscle, ovary, and thymus; at elevated levels in placenta, testis, and small intestine; and at high levels in adult kidney and stomach, which expressed CRTR-1 at levels 7- and 1.5-fold greater than ES cells, respectively. Expression of CRTR-1 was therefore specifically regulated in a temporal and spatial fashion both during embryogenesis and in the adult mouse.

Expression of CRTR-1 in Embryonic and Adult Kidney Is Restricted to the Distal Convoluted Tubules—Cellular localization of CRTR-1 expression was investigated in embryonic and adult kidneys. Wholemount in situ hybridization analysis was carried out using kidneys isolated from 16.5-d.p.c. embryos where CRTR-1 expression was demonstrated to be highest (Fig. 3B). Embryonic kidneys were probed with CRTR-1-specific sense and antisense digoxigenin-labeled riboprobes prior to embedding, sectioning, and counterstaining. Kidney sections probed with CRTR-1 sense control probe showed no specific staining (Fig. 4A). Kidney sections probed with CRTR-1 antisense probe showed specific staining representing CRTR-1 expression in the epithelial monolayer lining a subset of tubules in the embryonic kidney cortex (Fig. 4, B and C). CRTR-1-expressing tubules were identified as DCTs because they were located adjacent to glomeruli, consistent with the location of DCTs within the kidney cortex (42). Furthermore, only a small proportion of the tubules present in any cortical section expressed CRTR-1, consistent with the greater relative representation of proximal convoluted tubules in this region of the kidney (43–46). Finally, the morphology of CRTR-1-expressing tubules was clear and open, consistent with the morphology of DCTs but distinct from that of proximal convoluted tubules, in which the epithelium forms a brush border consisting of microvilli that project into the lumen of the tubule (45, 46). CRTR-1 expression was not detected in proximal convoluted tubules, glomeruli, or kidney vasculature (Fig. 4, B and C).

CRTR-1-expressing cells in the adult mouse kidney were determined by radiolabeled in situ hybridization to kidney sections because the greater volume of the adult kidney precludes the use of wholemount in situ hybridization. Adult kidney sections were sectioned and probed with CRTR-1-specific, [alpha-32P]labeled sense and antisense riboprobes. Hybridization was not detected using CRTR-1 sense control probe (Fig. 4, D and E). Adult kidney sections probed with CRTR-1 antisense probe showed specific localization of CRTR-1 expression to the epithelial monolayer lining a subset of tubules in the adult kidney cortex (Fig. 4, F and G). Consistent with the expression in embryonic kidneys, these tubules were identified as DCTs. CRTR-1 transcripts were not detected in the proximal convoluted tubules, glomeruli, or kidney vasculature (Fig. 4G). This analysis demonstrates that expression of CRTR-1 is spatially regulated in at least two distinct sites, the pluripotent cells of the developing mouse embryo and the epithelial cells lining the embryonic and adult kidney distal convoluted tubules.

CRTR-1 Acts as a Transcriptional Repressor in a Variety of Cell Types—Members of the CP2 family have been reported to act as transcriptional activators in both in vitro (1, 3) and in vivo (3, 8–11) transcription assays. The ability of CRTR-1 to act as a transcriptional regulator could not be investigated using target gene expression because the DNA binding sequence for this protein is unknown. The transcriptional activity of CRTR-1 was therefore assessed as a fusion protein with amino acids 1–174 of the Gal4 DNA binding domain (DBD) (28) in the plasmid pGalO-CRTR-1. pTK-MH100x4-LUC (36), which contains a luciferase gene regulated by the thymidine kinase (TK) promoter and four upstream tandem copies of the Gal4 binding site, was used as a reporter.

Plasmids were transfected into COS-1 cells, and levels of luciferase activity were analyzed in cell extracts 36 h post-transfection. Cotransfection of the Gal4-DBD, pGalO (23–27), with pTK-MH100x4-LUC did not alter the reproducible levels of luciferase activity (Fig. 5A, column 1) compared with pTK-MH100x4-LUC alone (Fig. 5A, column 2). Cotransfection of pGalO-CRTR-1 with pTK-MH100x4-LUC resulted in a 10–15-fold reduction in luciferase activity (Fig. 5A, column 3). CRTR-1-mediated transcriptional repression was also demonstrated in 293T (Fig. 5B) and ES cells (Fig. 5C), where expression of the Gal4-DBD-CRTR-1 fusion protein reduced luciferase expression 2.5- and 3.5-fold, respectively. This transcriptional repression was specific for the reporter plasmid pTK-MH100x4-LUC and not a result of general transcriptional toxicity of the Gal4-

| Name     | Origin   | Identity | Similarity | Reference |
|----------|----------|----------|------------|-----------|
| LBP-9    | Human    | 88       | 91         | 9         |
| CP2      | Mouse    | 68       | 79         | 2         |
| LBP-1c   | Human    | 67       | 79         | 2         |
| NFP3d9   | Mouse    | 62       | 78         | 4         |
| LBP-1a   | Human    | 61       | 76         | 8         |
| LBP-1d   | Human    | 60       | 71         | 8         |
| LBP-1b   | Human    | 57       | 71         | 8         |
| GRH      | Drosophila | 24°    | 40°        | 40        |
| CG11867  | Drosophila | 44      | 59         |           |

*a Percentage identity and similarity is over amino acids 632–865 of GRH.
FIG. 2. Multiple amino acid sequence alignment of CRTR-1. A, reported members of the CP2 family of transcription factors and the DNA binding domain (amino acids 632–865) of the D. melanogaster protein GRH. Dark shading indicates conservation of identical amino acids, whereas lighter shading indicates conservation of similar amino acids. B, schematic summary of conserved regions in CRTR-1 functionally important in LBP-1c (14) (conserved DNA binding domain (amino acids 45–366) and conserved oligomerization domain (amino acids 248–386)) and GRH (41) (conserved DNA binding domain (amino acids 45–260)). Also shown are the N-terminal 47 amino acids of CRTR-1 conserved only with LBP-9 (9).
A CP2-related, Developmentally Regulated Repressor

CRTR-1 Is a Novel Mouse Member of the CP2 Family of Transcription Factors—The CRTR-1 open reading frame was closely related to a group of proteins including the mouse transcription factor CP2 (Table I and Fig. 2) (2), the founding member of an expanding group of highly conserved proteins implicated in transcriptional control. Amino acid conservation across the CP2 open reading frame suggested conservation of functionally important regions of the CRTR-1 protein. In particular, a potential DNA binding domain, distinct from structurally characterized DNA binding domains, was identified between CRTR-1 amino acids 45 and 260, consistent with conservation of this region with the DNA binding domain of the melanogaster protein GRH (41) and deletion mapping of the LBP-1c DNA binding domain (14). Furthermore, a region implicated by deletion mapping in homo-oligomerization of LBP-1c (14) was highly conserved with CRTR-1 residues 261–386, suggesting that this protein is likely to support the formation of protein complexes. Whereas CRTR-1 shared greatest identity (88%) with the recently reported human protein LBP-9 (9), conservation was restricted to the open reading frame and did not extend into the 3′ untranslated region. This suggests that the N-terminal 52 amino acids of CRTR-1 are both necessary and sufficient for the transcriptional repression exerted through CRTR-1.

**DISCUSSION**

**The Ability of CRTR-1 to Repress Transcription Resides in an N-terminal Repression Domain—**The N-terminal 40 amino acids of CP2 have been shown to contain the CP2 transcriptional activation domain. This region of CRTR-1 was not conserved with members of the CP2 family reported to act as transcriptional activators but was closely related to LBP-9, which can antagonize LBP-1b-mediated transcriptional activation (9).

PCR was used to amplify the N-terminal 52 amino acids and the C-terminal 435 amino acids of CRTR-1. PCR products were cloned in frame with the GAL4-DBD in pGALO to generate pGALO-CRTR-1(1–52) and pGALO-CRTR-1(47–481), respectively. Cotransfection of COS-1 cells with pGALO-CRTR-1(1–52) and pTK-MH100x4-LUC did not affect levels of luciferase activity. Cotransfection of COS-1 cells with pGalO-CRTR-1(1–52) and pGalO-CRTR-1(47–481), respectively. Cotransfection of COS-1 cells with pGalO-CRTR-1(1–52) and pGalO-CRTR-1(47–481), respectively. Cotransfection of COS-1 cells with pGalO-CRTR-1(1–52) and pGalO-CRTR-1(47–481), respectively.

**CRTR-1 expression in embryonic and adult mouse kidneys.**—A-C, whole mount in situ hybridization of 16.5-d.p.c. mouse kidney probed with CRTR-1-specific sense (A) and antisense (B and C) digoxigenin-labeled riboprobes. D–G, radiolabeled in situ hybridization on 7-μm adult kidney sections using CRTR-1 sense (D and E) and antisense (F and G) [α-33P]UTP-labeled riboprobes. Developed slides were viewed under light (D and F) and dark field (E and G) condensers. D, distal convoluted tubule; G, glomerulus; P, proximal convoluted tubule. Magnifications are as follows: ×20 (A), ×20 (B), ×40 (C), ×10 (D), ×20 (E), ×20 (F), ×20 (G).

**Fig. 4.** CRTR-1 expression in embryonic and adult mouse kidneys. A–C, whole mount in situ hybridization of 16.5-d.p.c. mouse kidney probed with CRTR-1-specific sense (A) and antisense (B and C) digoxigenin-labeled riboprobes. D–G, radiolabeled in situ hybridization on 7-μm adult kidney sections using CRTR-1 sense (D and E) and antisense (F and G) [α-33P]UTP-labeled riboprobes. Developed slides were viewed under light (D and F) and dark field (E and G) condensers. D, distal convoluted tubule; G, glomerulus; P, proximal convoluted tubule. Magnifications are as follows: ×20 (A), ×20 (B), ×40 (C), ×10 (D), ×20 (E), ×20 (F), ×20 (G).

**Fig. 3.** CRTR-1 expression during later mouse development and in the adult mouse. Ribonuclease protection assays were carried out on 10 μg of total RNA isolated from (A) 10.5–17.5-d.p.c. mouse embryos, (B) tissues from the 18.5-d.p.c. mouse embryo, and (C) tissues from adult mice. mGAP antisense riboprobes were used as a loading control. SI, small intestine.

**A**

| CRTR-1 | 10.5 | 11.5 | 12.5 | 13.5 | 14.5 | 15.5 | 16.5 | 17.5 |
|--------|------|------|------|------|------|------|------|------|
| mGAP   |      |      |      |      |      |      |      |      |

**B**

| CRTR-1 | Brain | Intestine | Kidney | Liver | Lung | Skn | ES | mDNA |
|--------|-------|-----------|--------|-------|------|-----|----|------|
| mGAP   |       |           |        |       |      |     |    |      |

**C**

| CRTR-1 | Brain | Heart | Kidney | Liver | Lung | Skn | ES | mDNA |
|--------|-------|-------|--------|-------|------|-----|----|------|
| mGAP   |       |       |        |       |      |     |    |      |

*3 S. Jane, personal communication.*
either that the reported CRTR-1 and LBP-9 cDNAs are derived from alternative splicing of a homologous gene in mice and humans or that the proteins are not products of homologous genes. Sequence analysis therefore identified CRTR-1 as a novel mouse member of the CP2 family, with potential roles in transcriptional control and the formation of protein complexes.

**CRTR-1 Is a Novel Transcriptional Repressor**—CRTR-1 was shown to repress transcription when bound at a heterologous promoter. Whereas the extent of repression varied from 2.5- to 15-fold in different cell types (Fig. 5), conservation of this activity in different cell lines suggests that these results are indicative of normal CRTR-1 activity. This is supported by the fact that 293T and ES cells, in which repression was demonstrated, are representative of *in vivo* expression sites in kidney and pluripotent cells, respectively. The transcriptional repression activity of CRTR-1 was found to be localized to the N-terminal 52 amino acids, a region that does not show strong homology to other members of the CP2 family, with the exception of LBP-9, which has been shown to antagonize LBP-1b-mediated transcriptional activation by an unknown mechanism (9). The results presented here demonstrate that the N-terminal 52 amino acids of CRTR-1 are both necessary and sufficient for CRTR-1-mediated transcriptional repression when recruited to the promoter by DNA binding and that the observed transcriptional repression was not the result of steric hindrance caused by Gal4-DBD-CRTR-1 fusion proteins.

Activity as a transcriptional repressor distinguishes CRTR-1 from most other members of the CP2 family, which have been reported to act as transcriptional activators (1, 3, 8–11). This is consistent with the lack of amino acid conservation at the N terminus, which contains the activation domain in CP2 (4) and with the lack of polyglutamine- and glutamine/proline-rich sequences suggested as activation domains in the LBP-1c and CP2 sequences, respectively (2). LBP-9, identified as a sequence-specific binding protein on the −155/−131 region of the P450scc promoter, also exhibits unusual transcriptional activity. Whereas LBP-1b, which also binds this sequence, activated transcription of a linked reporter gene 21-fold in JEG-3 cells, LBP-9 did not activate transcription in the same system (9). Transfection of cells with increasing amounts of LBP-9 suppressed the LBP-1b-mediated reporter activation to basal levels. The mechanism of inhibition was not resolved and could result from direct repression of transcription, steric exclusion of LBP-1b from the DNA binding site, or displacement of LBP-1b from the promoter by formation of complexes with LBP-9. By contrast, CRTR-1 is the first reported CP2 family member that represses transcription directly from a heterologous promoter. Conservation of the 52-amino acid region of CRTR-1, responsible for transcriptional repression, with the equivalent region of LBP-9 may provide a mechanistic explanation for the suppression of LBP-1b-mediated transcription activation by LBP-9. In particular, if heteromeric complexes including LBP-9 can be localized at the P450scc promoter, this protein and possibly CRTR-1 may be capable of acting as a dominant repressor of promoters that are activated by CP2 family proteins. Resolution of this possibility requires identification of the CRTR-1 DNA binding sequence and binding partners.

Transcriptional repression can be mediated through several different mechanisms such as interference with assembly of the transcriptional machinery (47) or recruitment of corepressors including histone deacetylases (48). Protein sequence motifs present in DNA-binding transcriptional repressors that mediate interaction with corepressor proteins include PXXDLs in the ikaros protein (49–51), WRPW in Hairy-related bHLH proteins (52, 53), and a Gly/Arg-rich sequence present in the transcription factor YY1 (54). Furthermore, a histone deacetylase-independent mechanism of transcriptional repression has been described for methyl-CpG-binding protein 2 that is dependent on the presence of a conserved 30-amino acid sequence that contains two clusters of basic amino acids (55). These motifs could not be identified within the N-terminal 52 amino acids of CRTR-1, suggesting a novel mechanism of transcriptional repression for this protein. Conservation of repressor activity in cell lines of diverse origin and properties such as ES cells, 293T cells, and COS-1 cells suggests that factors required for CRTR-1-mediated repression are widely expressed.

**Expression of CRTR-1 Is Spatially and Temporally Regulated during Mouse Development**—Expression of CRTR-1 was shown to be spatially and temporally regulated, both during embryogenesis and in the adult mouse. *In vitro*, CRTR-1 was expressed in ES cells and rapidly down-regulated upon differ-
entiation to EPL cells (Fig. 1, B and C). An equivalent expression pattern has been described in vivo where CRTR-1 expression occurs in pluripotent cells of 3.5-d.p.c. mouse embryos is down-regulated at around 4.75 d.p.c.2 Re-expression of CRTR-1 in experiments with kidney and placenta, but expression was not detected in adult liver. Whereas direct parallels cannot be drawn between expression in kidney DCTs, which comprise only a small proportion of the cells within the kidney, suggesting important CRTR-1 function at this location. DCTs arise from the metanephric mesenchyme, which is located near the cortical periphery after 13 d.p.c. Although CRTR-1 expression in the embryo may be associated with induction of these tubules during development, continued high level expression of the gene at later stages of embryogenesis and in the adult is suggestive of a role for CRTR-1 in DCT function and physiology. Resolution of the functional relevance of CRTR-1 expression at different locations and developmental stages awaits functional investigation.

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