

# Identification of a Short *cis*-Acting Element in the Human Vasopressin Type 2 Receptor Gene Which Confers High-Level Expression of a Reporter Gene Specifically in Collecting Duct Cells

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In the kidney, water reabsorption is mainly regulated by the binding of arginine vasopressin to vasopressin type 2 (V2) receptors. These receptors are expressed selectively in principal cells of the collecting ducts. To identify molecular mechanisms responsible for the cell-specific expression of the V2 receptor, we have analyzed the proximal promoter of the corresponding gene. We report the identification of a 33-bp enhancer [collecting duct tissue-specific element 1 (CSE1)] that induced high levels of expression of the luciferase reporter gene in three collecting duct cell lines, but not in other renal cell lines. In gel shift assays, CSE1 bound a DNA-binding protein expressed selectively in collecting duct cell lines, and a 7-bp mutation, which abolished the activity of CSE1 in transient transfection experiments, also abolished the binding of this protein. Furthermore, decoy experiments performed using CSE1 showed that this sequence was involved not only in the expression of a construct containing 4.2 kb of the V2 receptor proximal promoter, but also in the expression of the endogenous V2 receptor gene. CSE1 appears to act mostly by counteracting the inhibitory effects of a strong ubiquitous repressor element that we called CIE1. Collectively, these results identify the first functional collecting duct-specific *cis*-acting element. (*Molecular Endocrinology* 14: 1682-1695, 2000)

## INTRODUCTION

One of the major roles of the kidney is to maintain the homeostasis of body fluids by regulating the elimination of ions and water. Regulation of water elimination takes place in collecting ducts, which form the most distal part of the nephrons and which are composed of three different cell types: intercalated cells  $\alpha$ , intercalated cells  $\beta$ , and principal cells (reviewed in Ref. 1). Intercalated cells are implicated in acid-base homeostasis, while principal cells, which are by far the most abundant ones, are involved in potassium secretion, sodium reabsorption, and water reabsorption. Water reabsorption is mostly regulated by arginine vasopressin (AVP), a hormone produced in the pituitary gland. AVP binds to vasopressin type 2 (V2) receptors, resulting in an increase of adenylate cyclase activity and promoting the cAMP-mediated incorporation of water channels into the luminal surface of principal cells (reviewed in Ref. 2). The importance of V2 receptors in the regulation of urine concentration is illustrated by the consequences of mutations in the coding sequence of the corresponding gene. Such mutations are responsible for nephrogenic diabetes insipidus, a disease that is characterized by an inability to concentrate urine, leading to polyuria and polydipsia, and which can be responsible for repeated episodes of dehydration in early infancy, and thus for mental retardation (reviewed in Ref. 2).

The V2 receptor is a polypeptide of 371 amino acids, with a structure typical of G protein-coupled receptors, which contain seven transmembrane domains (3, 4). It is encoded by a gene located on the X chromosome, which is formed by three exons separated by two short introns (5). In different mammalian species, Northern blot analyses have shown that the expression of the V2 receptor gene is restricted to the kidney (3, 4). *In situ* hybridization

experiments and immunohistochemical studies have confirmed this restricted pattern of expression. They have shown that, in the kidney, the V2 receptor gene is expressed predominantly by principal cells of the collecting ducts. It is also expressed at lower levels by some cells of the thick ascending limb of the loop of Henle, while it is not expressed by other renal cells (6–8). Furthermore, during embryonic development, *in situ* hybridization studies performed in rat have shown that expression of the V2 receptor gene begins around day 16 post conception, *i.e.* at a time corresponding to the formation of the permanent collecting ducts, and that its expression is restricted to collecting ducts (7). The V2 receptor gene can thus be considered as a good model for studying collecting duct-specific transcription mechanisms.

Two rabbit collecting duct cell lines have been generated in our laboratory (9, 10), providing us with very useful tools to study the expression of the V2 receptor gene. These cells have been obtained by infecting primary cultures of renal cortical cells with either wild-type SV40 strain (RC.SV3A2 cells) or with a temperature-sensitive mutant strain (RC.SVtsA58 cells) (9, 10). RC.SVtsA58 cells, which are the most extensively characterized, have typical features of principal cells of the collecting ducts. They express the V2 receptor and they are responsive to AVP, which induces a dose-dependent increase in the levels of cAMP; they are responsive to bradykinin, to atrial natriuretic peptide, and to prostacyclin  $E_2$ , they take up sodium via an amiloride-sensitive sodium channel, and they express the cell adhesion molecule L1 (10–12). RC.SV3 cells have also characteristic features of principal cells of the collecting ducts. They respond to AVP, which increases the production of cAMP, and they express the cell adhesion molecule L1 (9, 11).

To delineate molecular mechanisms responsible for the cell-specific expression of the human V2 receptor gene, we performed transfection experiments and DNA-binding assays, using different segments of the V2 receptor proximal promoter. We report the identification of a 33-bp enhancing sequence that strongly increases promoter activity selectively in collecting duct cell lines and which binds a nuclear protein present only in these cell lines. Furthermore, in decoy experiments, this element down-regulates the expression of the endogenous V2 receptor gene. To give rise to a tissue-specific expression, this short tissue-specific sequence appears to act mostly by counteracting the effects of a strong repressor element.

## RESULTS

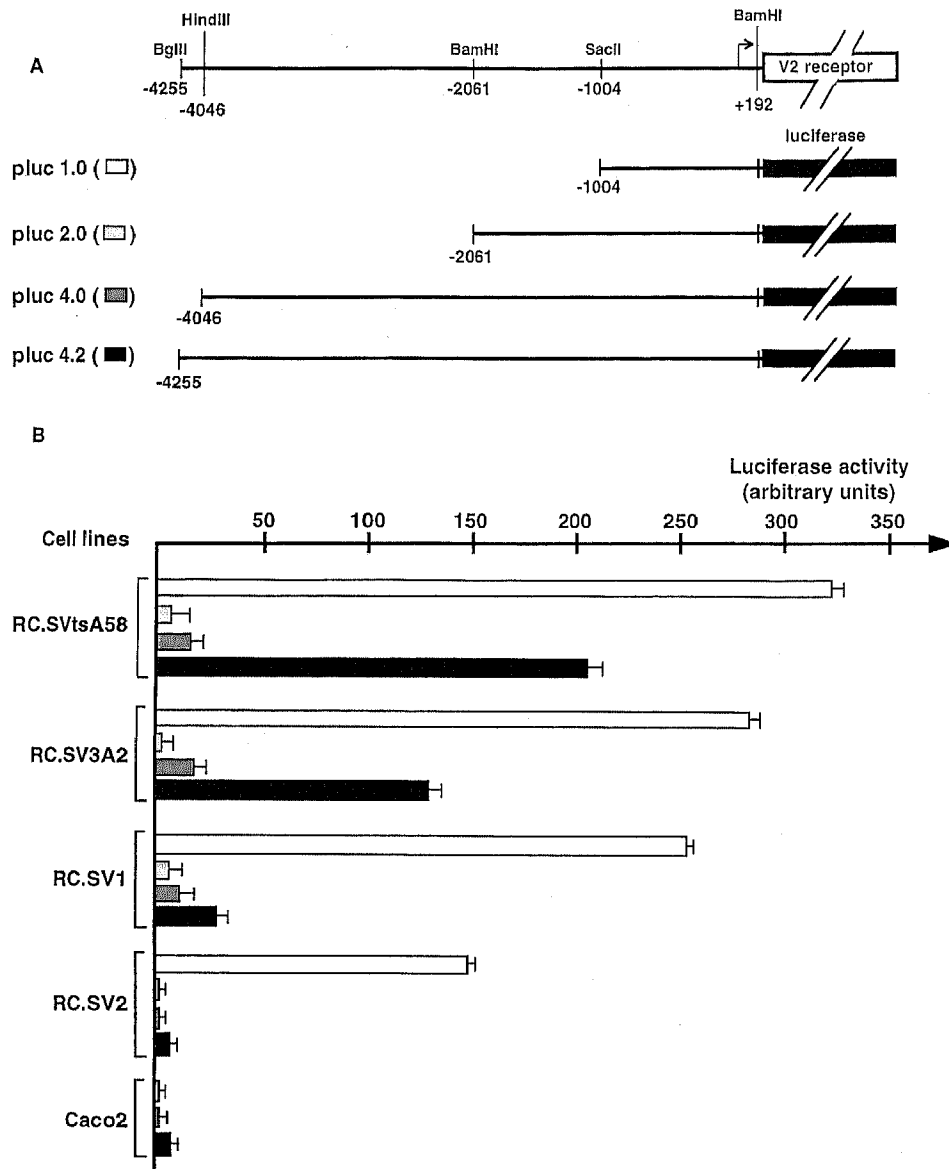
### Identification of a Segment of the V2 Receptor Promoter That Is Active Selectively in Collecting Duct Cell Lines

Our first goal was to delineate a segment of the V2 receptor proximal promoter able to drive reporter gene activity in collecting duct cell lines, but not in other cell

lines. To do so, we performed transient transfection experiments using segments of the V2 receptor proximal promoter of increasing length, cloned upstream of the luciferase reporter gene. The 3'-end of all these promoter segments extended up to a *Bam*HI site located 230 bp upstream of the translation initiation codon, which was known to be downstream of the transcription start site (3, 5, 13). These constructs were transfected in four different rabbit cell lines derived either from collecting ducts (RC.SVtsA58 cells and RC.SV3A2 cells), or from other segments of the nephron (RC.SV1 cells and RC.SV2 cells), and in Caco 2 intestinal cells. The V2 receptor gene was known to be expressed in RC.SVtsA58 cells, and in RC.SV3A2 cells but not in RC.SV1 cells, in RC.SV2 cells, and in Caco 2 cells (9–12).

We first cloned a 1.0-kb fragment of the V2 receptor proximal promoter upstream of the luciferase reporter gene (pluc 1.0) (Fig. 1A). The transgene was active at high levels in all four rabbit cell lines (Fig. 1B), which suggested that this 1.0-kb fragment contains a strong ubiquitous enhancer, but no cell-specific element. A 2.0-kb segment of the V2 receptor proximal promoter was then cloned upstream of the luciferase gene (pluc 2.0) (Fig. 1A). pluc 2.0 was active at very low levels in all four renal cell lines, as well as in Caco 2 cells (Fig. 1B). It was at least 35-fold less active than pluc 1.0 in all four renal cell lines (Fig. 1B), and its expression level was similar to a construct containing only 47 bp of the mouse  $\beta$ -globin minimal promoter (pluc 47G) (data not shown). These results showed that the segment of the V2 receptor promoter extending from  $-2.0$  kb to  $-1.0$  kb contains a very strong negative regulatory element, which was able to inhibit the activity of pluc 1.0 in all renal cell lines. We called this element CIE1 (for collecting duct inhibitory element 1).

A construct harboring a segment of the V2 receptor proximal promoter extending up to  $-2.2$  kb (pluc 2.2) had the same activity as pluc 2.0 in all cell lines (data not shown). Similarly, when a 4.0-kb fragment of V2 receptor proximal promoter was cloned upstream of the luciferase gene (pluc 4.0), it was active at very low levels in all cell lines (Fig. 1). On the contrary, a transgene containing a 4.2-kb segment of the V2 receptor proximal promoter cloned upstream of the luciferase gene (pluc 4.2) was much more active in collecting duct cell lines than in other renal cell lines or in Caco 2 cells (Fig. 1). Furthermore, pluc 4.2 was only 4.3 and 3.5 times more active than pluc 2.0 in RC.SV1 cells and in RC.SV2 cells, respectively, but it was 25 and 30 times more active in RC.SVtsA58 cells and in RC.SV3A2 cells, respectively. pluc 4.2 was also active at high levels when it was stably transfected in RC.SV3A2 cells (data not shown). These results showed that a 4.2-kb segment of the V2 receptor proximal promoter was able to induce a cell-specific expression of the reporter gene in transient transfection experiments and suggested that the *cis*-acting element responsible for this cell-specific expression lies between  $-4.2$  kb and  $-4.0$  kb. They also sug-



**Fig. 1.** Functional Analysis of Different Segments of the V2 Receptor Proximal Promoter

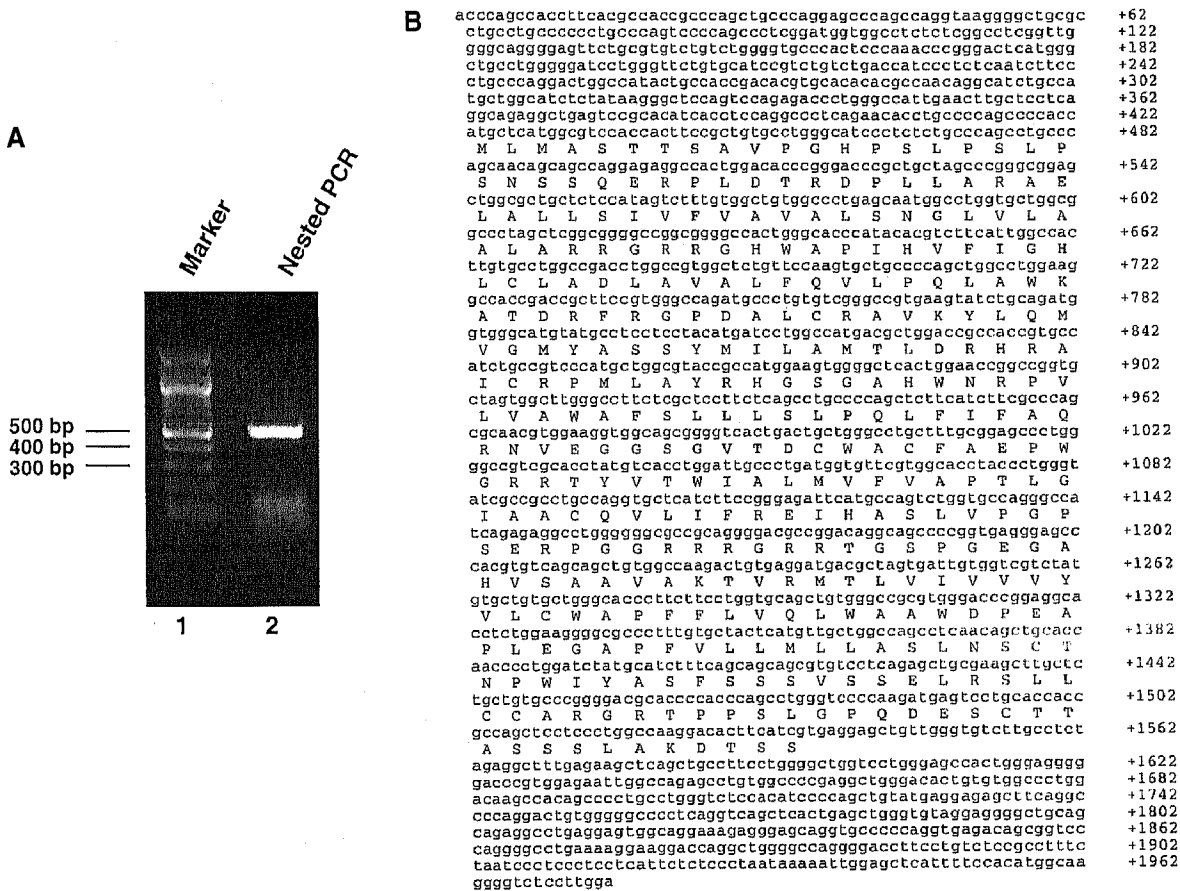
A, Schematic representation of the constructs used for transfection experiments. B, Results of transient transfection experiments performed using the constructs depicted in panel A. These constructs were transfected in two collecting duct cell lines (RC.SVtsA58 cells and RC.SV3A2 cells), in two epithelial cell lines derived from other segments of the nephron (RC.SV1 cells and RC.SV2 cells), and in an epithelial intestinal cell line (Caco 2 cells). pSV- $\beta$ -galactosidase control vector was used to correct for transfection efficiency. Values represent the mean  $\pm$  SD. Experiments were repeated at least three times in triplicate.

gested that this tissue-specific *cis*-acting element acts mostly by counteracting the inhibitory effects of CIE1.

#### Mapping of the Transcription Start Site of the V2 Receptor Gene

Before studying in detail the promoter fragment delineated above, the transcription start site of the human V2 receptor gene was mapped. To obtain cDNA sequences corresponding to the full-length 5'-end of the V2 receptor mRNA, 5'-RACE (rapid amplification of cDNA ends) experiments were performed using total

RNA from RC.SV3A2 cells stably transfected with pluc 4.2, and primers specific of the luciferase gene. After two rounds of PCR, a single band of about 550 bp could be detected by electrophoresis of the PCR products (Fig. 2A). This band was cloned in pGEM-T, and all of the 36 different clones tested contained an insert of the same size. Four of these clones were sequenced, and all of them contained an identical sequence, corresponding to a transcriptional start site located 422 bp upstream of the translation initiation codon (Fig. 2B). Analysis of the sequence located immediately upstream of the transcription start site in



**Fig. 2.** Identification of the Transcriptional Start Site of the V2 Receptor Gene

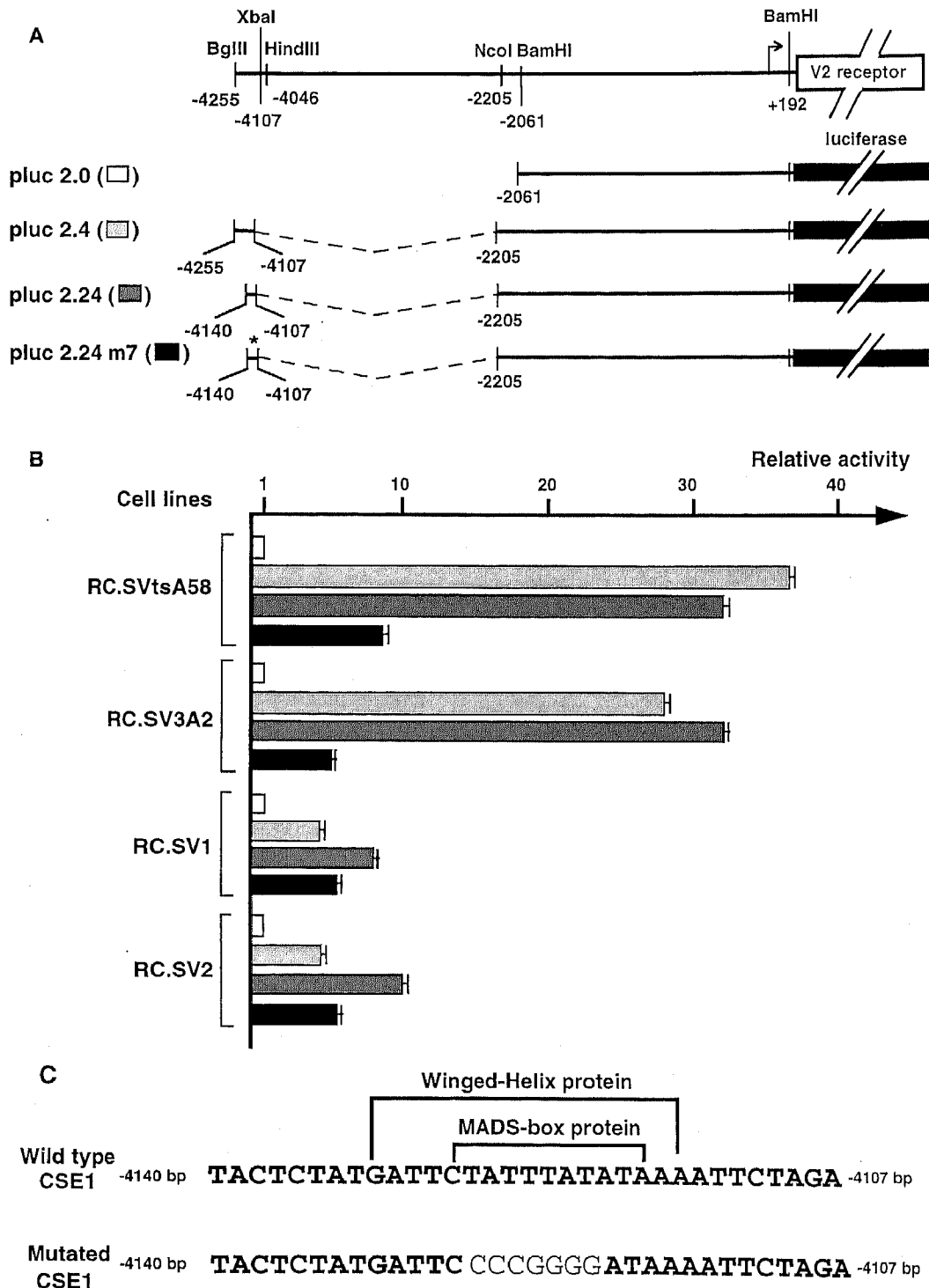
A, Agarose gel electrophoresis of the PCR products obtained in a 5'-RACE assay. Total RNA from RC.SV3A2 cells stably transfected with pluc 4.2 was used as a template to synthesize first strand cDNA, in the presence of a primer specific of the luciferase gene. After labeling this first strand cDNA using dCTP and the terminal deoxynucleotidyl transferase, two rounds of PCRs were performed using primers specific of the 5'-tail and of the luciferase gene. The nested PCR products were electrophoresed in an agarose gel and stained with ethidium bromide. Lane 1, 100-bp ladder (Life Technologies, Inc.); lane 2, a single PCR product of approximately 550 bp was obtained. B, Complete sequence of the human V2 receptor mRNA, and of the corresponding protein. It was deduced from the sequence of the PCR product shown in panel A, and from sequences already available (GenBank accession number NM 000054).

the V2 receptor gene (GenBank accession number U52111) confirmed that the V2 receptor promoter is a TATA-less promoter (data not shown).

**Identification of a Short Cell-Specific *cis*-Acting Element within the V2 Receptor Promoter**

To delineate more precisely the cell-specific element located between -4.2 kb and -4.0 kb in the V2 receptor promoter, we looked for a minimal sequence that would be able to increase the activity of the luciferase reporter gene specifically in RC.SVtsA58 cells and in RC.SV3A2 cells, when cloned upstream of the 2.2 kb promoter segment in pluc 2.2. The activity of these constructs was compared with the activity of pluc 2.0, since it was almost inactive in all four renal cell lines (Fig. 1). First, a 148-bp sequence extending from -4,255 bp to -4,107 bp was cloned upstream of the V2 receptor proximal promoter in pluc 2.2 (pluc 2.4) (Fig. 3A). pluc 2.4 was 4 times more active than

pluc 2.0 in RC.SV1 cells and in RC.SV2 cells, but it was 37 and 28 times more active than this latter construct in RC.SVtsA58 cells and in RC.SV3A2 cells, respectively (Fig. 3B), which indicated that the -4,255-bp to -4,107-bp segment contains a *cis*-acting element that could enhance the expression of the reporter gene specifically in collecting duct cell lines. Computer-based analysis of this 148-bp sequence showed that its 3'-end contains a potential binding site for winged-helix transcription factors and another one for MADS-box transcription factors related to MEF-2, while its 5'-end contains mostly binding sites for ubiquitous transcriptions factors such as Sp1 and AP-1 (14). Winged-helix proteins and MADS-box transcription factors being involved in cell-type-specific gene expression and in cell differentiation, we focused our attention on the most 3'-part of the 148-bp segment. A 33-bp sequence located between -4,140 bp and -4,107 bp was cloned upstream of the V2 receptor proximal promoter in pluc 2.2 (pluc 2.24) (Fig. 3A), and



**Fig. 3.** Identification of a *cis*-Acting Element Active Selectively in Collecting Duct Cell Lines

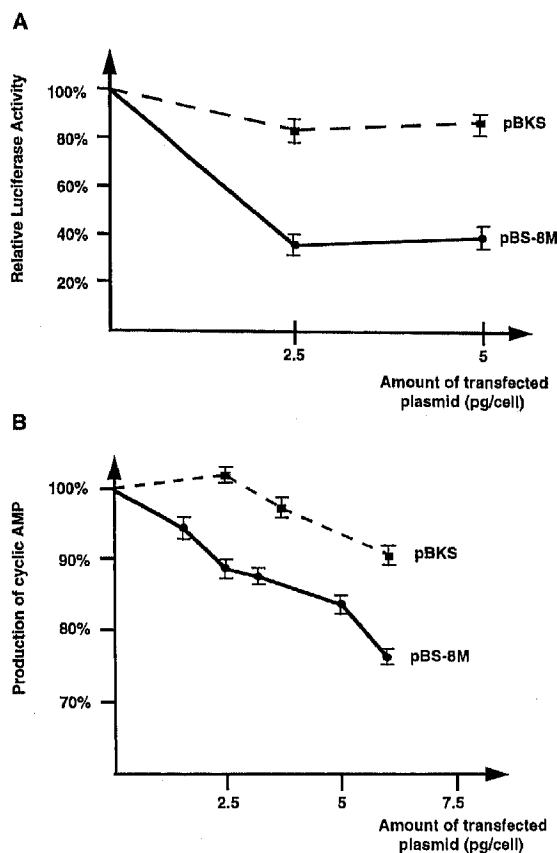
A, Schematic representation of the different constructs used for transfection experiments. B, Results of transient transfection experiments performed using the constructs depicted in panel A. Each construct was transfected in two collecting duct cell lines (RC.SVtsA58 cells and RC.SV3A2 cells), and in two epithelial cell lines derived from other segments of the nephron (RC.SV1 cells and RC.SV2 cells). pSV- $\beta$ -galactosidase control vector was used to correct for transfection efficiency. Results are expressed as a ratio between the activity of the tested construct and the activity of pluc 2.0. C, Sequence of the 33-bp segment that is located between -4,140 bp and -4,107 bp, and which was used to construct pluc 2.24 (wild-type CSE1). This sequence contains a potential binding site for winged-helix proteins, and another one for MADS-box proteins related to MEF-2. Sequence of a segment containing a 7-bp mutation (*light-face letters*) and used to construct pluc 2.24 m7 (mutated CSE1). Values represent the mean  $\pm$  SD. Experiments were repeated at least three times in triplicate.

this construct was used in transfection experiments. pluc 2.24 had the same activity as pluc 2.4 in RC.S-VtsA58 cells and in RC.SV3A2 cells (Fig. 3B), confirming that the enhancer element is located within this 33-bp segment. It is of note that pluc 2.24 was about 2 times more active than pluc 2.4 in control cell lines (Fig. 3B). A 7-bp mutation was then introduced in the 33-bp sequence, and the mutated construct was cloned upstream of the V2 receptor proximal promoter in pluc 2.2 (pluc 2.24 m7) (Fig. 3A). This mutation was located within the potential binding sites for winged-helix proteins and for MADS-box proteins (Fig. 3C). The mutated construct was almost as active as pluc 2.24 in RC.SV1 cells and RC.SV2 cells (Fig. 3B). On the contrary, in the two collecting duct cell lines, its activity was dramatically decreased when compared with pluc 2.24, and it was similar to the one observed in control cell lines (Fig. 3B). These results strongly suggested that the 33-bp sequence located between  $-4,140$  bp and  $-4,107$  bp contains a tissue-specific element that we called CSE1 (for collecting duct tissue-specific element 1).

To confirm that CSE1 is a positive regulatory element within the context of 4.2 kb of the V2 receptor proximal promoter, decoy experiments were performed, using a plasmid containing eight copies of CSE1 (pBS-8M). pBluescript KS containing no insert (pBKS) was used as a negative control, and pBS-8M or pBKS was transiently transfected in RC.SV3A2 cells that had been stably transfected with pluc 4.2. pBS-8M reduced the activity of pluc 4.2 by more than 50%, when compared with pBKS (Fig. 4A). The same approach was then used to determine whether CSE1 plays a role in the regulation of the expression of the endogenous V2 receptor gene. We used RC.SVtsA58 cells, since these cells express the V2 receptor and respond to AVP at a restrictive temperature (39.5 C) but not at a permissive temperature (33 C) (10); these phenotypical modifications were due to modification of mRNA stability (R. Piedagnel, unpublished results). Different amounts of pBS-8M or of pBKS were transiently transfected in RC.SVtsA58 at 33 C, and, after 3 h, the cells were switched at 39.5 C to induce the expression of V2 receptors at the cell surface. After 2 days, the production of cAMP in response to AVP was assessed. Transfection with increasing amounts of pBS-8M induced a reduction of the production of cAMP and for the highest amounts of pBS-8M, this reduction was as high as 18%, when compared with pBKS (Fig. 4B).

#### CSE1 Can Act as a Cell-Specific Enhancer

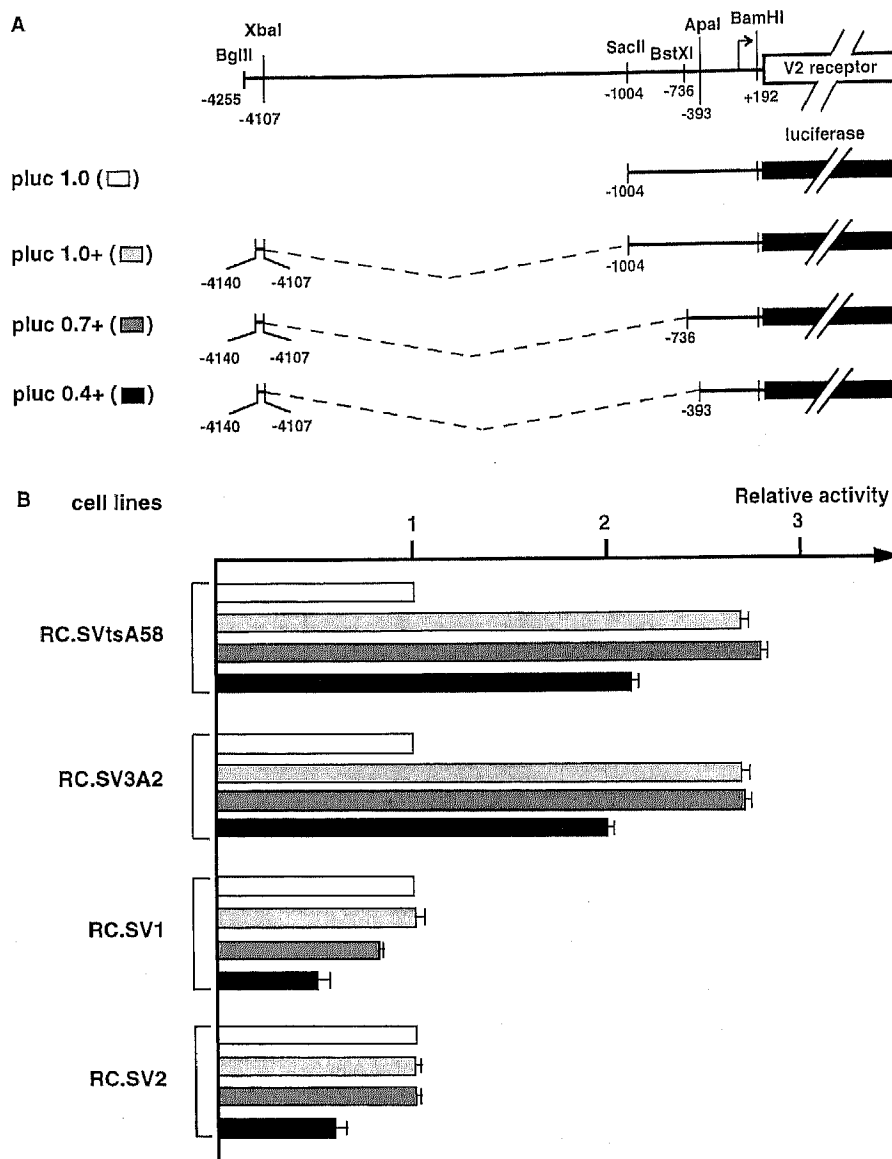
Transfection experiments performed using pluc 1.0, pluc 2.0, pluc 2.2, pluc 4.0, and pluc 4.2 suggested that CSE1 acts mostly by relieving the inhibitory effect of CIE1 (Fig. 1). To test the ability of CSE1 to directly enhance the activity of the V2 receptor proximal promoter, transfection experiments were performed using a construct containing CSE1 but not CIE1. CSE1 was



**Fig. 4.** Study of the Role of CSE1, Using Decoy Experiments

**A**, RC.SV3A2 collecting duct cells stably transfected with pluc 4.2 were transiently transfected either with a decoy plasmid containing eight copies of CSE1 (pBS-8M) or with a plasmid containing no insert (pBKS). Transfections were performed using either 2.5 pg or 5 pg of plasmid per cell. The luciferase activity was arbitrarily defined as 100% when RC.SV3A2 cells stably transfected with pluc 4.2 were not cotransfected. **B**, RC.SVtsA58 cells were transfected with either a decoy plasmid containing eight copies of CSE1 (pBS-8M) or a plasmid containing no insert (pBKS). Transfections were performed using 1.5 pg, 2.5 pg, 3.0 pg, 3.5 pg, 5 pg, 6.0 pg of plasmid per cell. The cAMP production was arbitrary defined at 100% when RC.SVtsA58 were not transfected. Values represent the mean  $\pm$  SD. Experiments were repeated at least three times in triplicate.

cloned upstream of a 1.0-kb segment of the V2 receptor proximal promoter (pluc 1.0+), and the activity of pluc 1.0+ was compared with the activity of a construct containing only a 1.0-kb segment of the V2 receptor proximal promoter (pluc 1.0) (Fig. 5A). pluc 1.0+ was about 3 times more active than pluc 1.0 in the two collecting duct cell lines (RC.SV3A2 cells and RC.SVtsA58 cells), but its activity was identical to the activity of pluc 1.0 in the two other renal cell lines (RC.SV1 cells and RC.SV2 cells) (Fig. 5B). The luciferase activities observed when the four cell lines were transfected with pluc 1.0+ were very similar to the ones detected when these cell lines were transfected with a construct containing CSE1 cloned upstream of



**Fig. 5.** Study of the Enhancing Activity of CSE1

A, Schematic representation of the different constructs used for transfection experiments. B, Transient transfection experiments were carried out in two collecting duct cell lines (RC.SVtsA58 cells and RC.SV3A2 cells) and in two epithelial cell lines derived from other segments of the nephron (RC.SV1 cells and RC.SV2 cells), using the constructs depicted in panel A. pSV- $\beta$ -galactosidase control vector was used to correct for transfection efficiency. Results are expressed as a ratio between the activity of the tested construct and the activity of pluc 1.0. Values represent the mean  $\pm$  sd. Experiments were repeated at least three times in triplicate.

a 736-bp segment of the V2 receptor proximal promoter (pluc 0.7+). They were only slightly higher than the luciferase activities observed when the cell lines were transfected with a construct containing CSE1 cloned upstream of a 393-bp segment of the V2 receptor proximal promoter (pluc 0.4+) (Fig. 5). Conversely, when CSE1 was cloned upstream of a segment of the V2 receptor promoter extending from -10 bp to +192 bp (pluc 0.05+), it was almost inactive in all cell lines (data not shown).

#### Identification of the Proteins Binding to CSE1

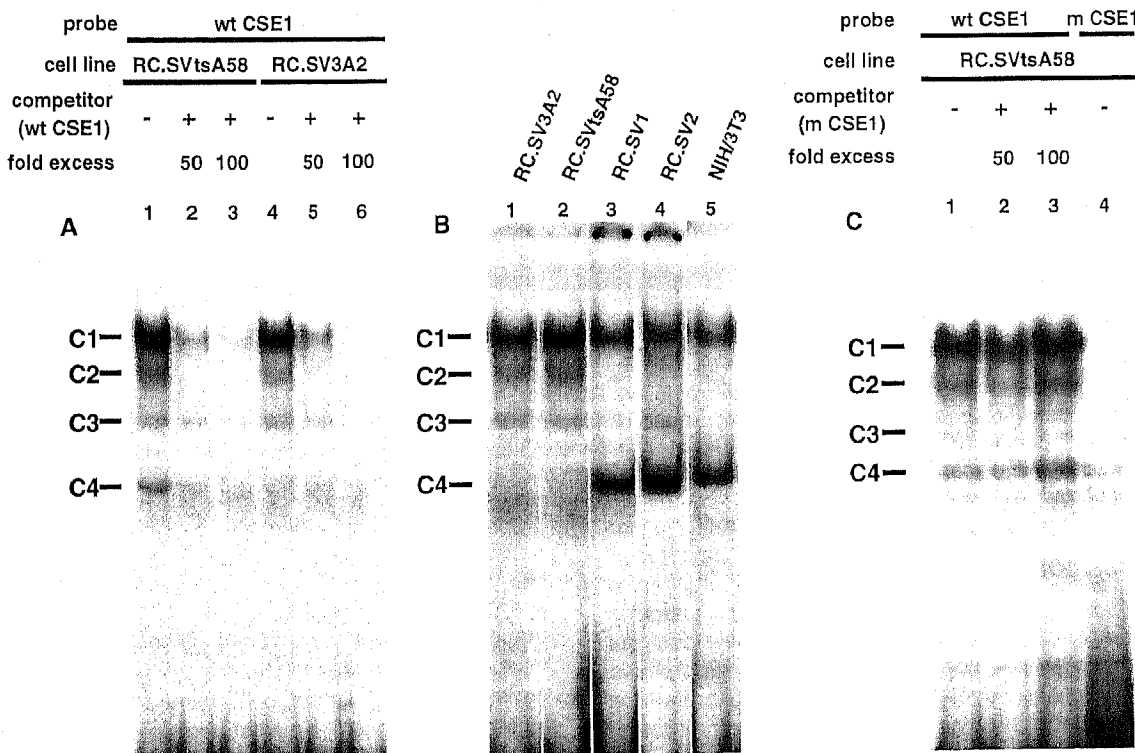
To identify the DNA-binding proteins binding to CSE1, gel shift experiments were performed using a double-stranded oligonucleotide extending from -4,140 bp to -4,107 bp as a probe, and nuclear extracts obtained from all four renal cell lines and from NIH/3T3 cells. When RC.SVtsA58 cells or RC.SV3A2 cells were used as a source of nuclear extracts, four DNA-protein complexes (complexes C1-C4) were observed, and all

these complexes were competed by a 50- to 100-fold molar excess of cold oligonucleotide (Fig. 6A). In contrast, when gel shift experiments were performed using nuclear extracts from RC.SV1 cells, RC.SV2 cells, or NIH/3T3 cells, a different pattern of DNA-protein complexes was observed. Specifically, complex C2 was not seen with any of these three nuclear extracts, and complex C3 was seen only when nuclear extracts from RC.SV2 cells were used (Fig. 6B). When a double-stranded oligonucleotide containing a 7-bp mutation identical to the one used to construct pluc 2.55 m7 (Fig. 3) was employed as cold competitor, it did not modify the pattern of DNA-protein complexes observed with nuclear extracts from RC.SVtsA58 cells (Fig. 6C) or from RV.SV3A2 cells (data not shown). In particular, the formation of the complex C2 was not inhibited. When this mutated oligonucleotide was used as a probe, complexes C1, C2, and C3 could not be detected with nuclear extracts from RC.SVtsA58 cells (Fig. 6C), or from RV.SV3A2 cells (data not shown). Hence, our DNA-binding assays suggest that a protein specific for principal cells of the collecting

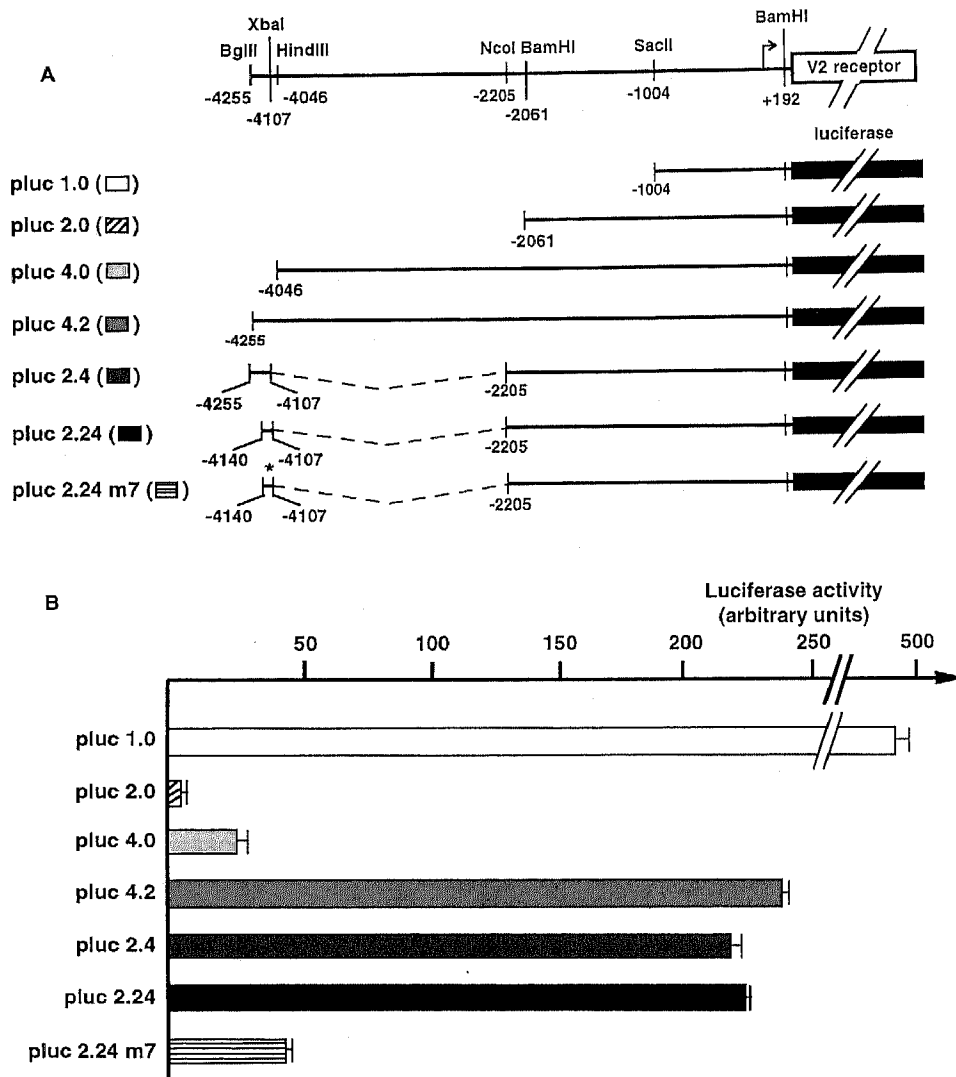
ducts binds to CSE1 (corresponding to complex C2), and not to a mutated sequence that has no cell-specific activity in transfection experiments.

**Transfection of a Human Collecting Duct Cell Line**

Results described above were obtained using rabbit renal cell lines. To test the relevance of our results in human collecting duct cells, we performed transfection experiments using a human collecting duct cell line (HCD.A8 cell line). The results obtained using this cell line were essentially the same as the ones obtained using the two rabbit collecting duct cell lines (RC.SVtsA58 cells and RC.SV3A2 cells). In particular, pluc 2.0, pluc 4.0 and pluc 2.24m7 were expressed at very low levels, while pluc 1.0, pluc 4.2, and pluc 2.24 were expressed at much higher levels (Fig. 7). The expression level of pluc 1.0+ was also 6 times higher than the expression level of pluc 1.0, confirming the enhancing activity of CSE1 in human collecting duct cells (data not shown).



**Fig. 6.** Electrophoretic Mobility Shift Analysis of the Proteins Binding to the 33-bp Sequence Extending from -4,140 bp to -4,107 bp (wt CSE1)  
 A, Lane 1 and lane 4 represent the different DNA-protein complexes observed using nuclear extracts from RC.SVtsA58 cells and from RC.SV3A2 cells. In lanes 2, 3, 5, and 6, competition assays were performed using a 50- and 100-fold molar excess of unlabeled oligonucleotide. B, DNA-protein complexes observed using nuclear extracts from five different cell lines. Complex C2 is only seen with nuclear extracts derived from collecting duct cell lines (lanes 1 and 2). C, Lane 1 represents the different DNA-protein complexes observed using nuclear extracts from RC.SVtsA58 cells and wt CSE1 as a probe. In lanes 2 and 3, competition assays were performed using a 50- to 100-fold molar excess of unlabeled oligonucleotide harboring a 7-bp mutation (m CSE1). Lane 4 represents the different DNA-protein complexes observed using nuclear extracts from RC.SVtsA58 cells and m CSE1 as a probe.



**Fig. 7.** Functional Analysis of Different Segments of the V2 Receptor Proximal Promoter in a Human Collecting Duct Cell Line  
A, Schematic representation of the different constructs used for transfection experiments. B, Results of transient transfection experiments performed using the constructs depicted in panel A. Each construct was transfected in HCD.A8 cells, and pSV- $\beta$ -galactosidase control vector was used to correct for transfection efficiency. Values represent the mean  $\pm$  SD. Experiments were repeated at least three times in triplicate.

## DISCUSSION

In kidney, V2 receptors are expressed selectively in differentiated principal cells of the collecting ducts (6–8), and the regulation of water reabsorption is mostly due to the binding of AVP to these receptors. With the long-term goal of identifying DNA-binding proteins responsible for the cell-specific expression of the human V2 receptor gene, we have analyzed regulatory sequences of the corresponding proximal promoter.

The coding sequence of the human V2 receptor gene has been extensively studied, since mutations that lead to the synthesis of an abnormal protein are responsible for X-linked nephrogenic diabetes insipidus (reviewed in Ref. 2), but the V2 receptor promoter

has not been studied in detail. In particular, the transcription start site of this gene has not been precisely mapped, although sequencing of cDNAs obtained from cells transfected with the human V2 receptor gene or from malignant cells have shown that it is located at least 235 bp upstream of the translation initiation codon (3, 5, 13). By performing 5'-RACE experiments with cells stably transfected with a construct containing more than 4 kb of the human V2 receptor proximal promoter, we have shown that the transcription start site is located 422 bp upstream of the translation initiation codon. Previous studies performed with the rat gene have shown that, in this species, the transcription start site is also located 422 bp upstream of the translation initiation codon (15). Using this transcription start site, the deduced length

of the human V2 receptor mRNA is 1,976 bp, which corresponds precisely to the size of the mRNA determined by Northern blot analyses in different species, including human (3).

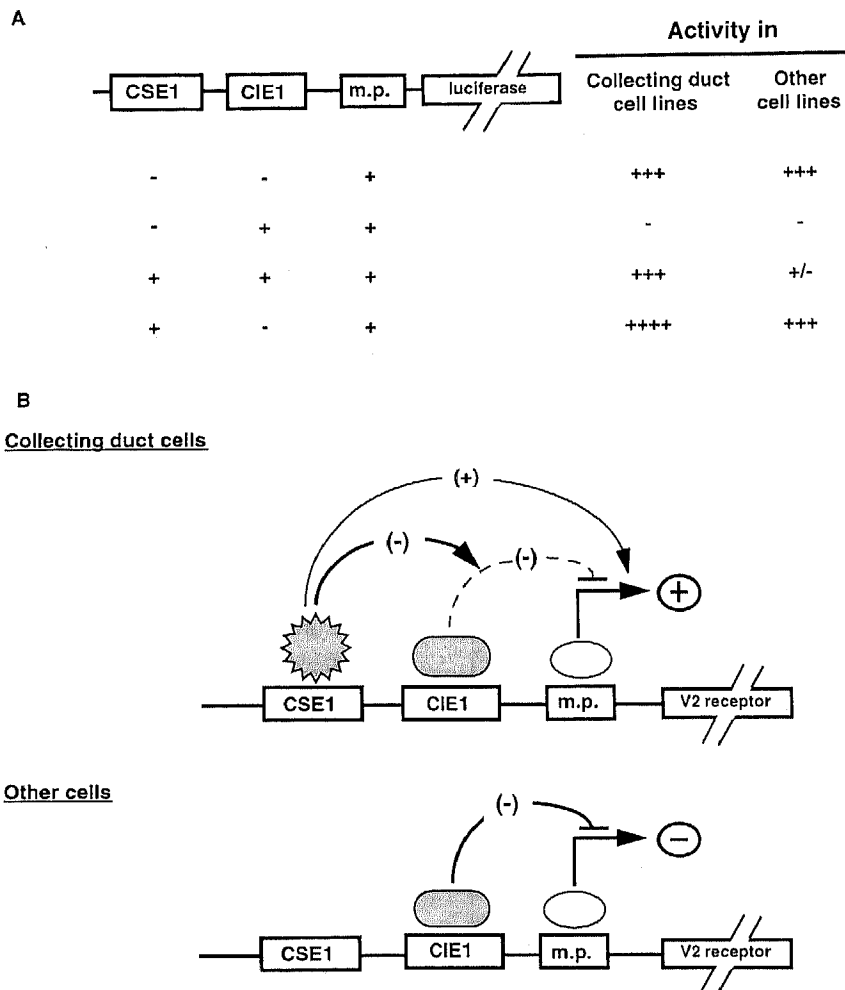
By transfecting constructs containing different segments of the V2 receptor proximal promoter in four rabbit renal cell lines, in a human collecting duct cell line and in a human intestinal cell line, we have identified a 33-bp sequence located between -4,140 bp and -4,107 bp, which was able to induce high-level expression of the reporter gene specifically in three different collecting duct cell lines. When this sequence, named CSE1, was cloned upstream of a 2.2-kb segment of the V2 receptor proximal promoter, which had almost no activity by itself, it induced high levels of expression of the reporter gene in three collecting duct cell lines (RV.SVtsA58 cells, RC.SV3A2 cells, and HCD.A8 cells), but not in cell lines derived from other segments of the nephron (RC.SV1 cells and RC.SV2 cells). A 7-bp mutation in CSE1 abolished the cell-specific activation of the reporter gene, the levels of expression of the mutated construct being similar in collecting duct cell lines and in control cell lines. Furthermore, when RC.SV3A2 cells were stably transfected with a construct containing 4.2 kb of the V2 receptor proximal promoter cloned upstream of the luciferase gene (pluc 4.2), transient transfection of these cells with a decoy plasmid containing eight copies of CSE1 induced a 2-fold reduction in the levels of expression of the reporter gene. Similarly, transfection of RC.SVtsA58 cells with the decoy plasmid induced a significant decrease in the production of cAMP in response to AVP and thus very probably inhibited the expression of the V2 receptor gene. These results were supported by gel shift assays, which showed that CSE1 bound a DNA-binding protein present in cell lines derived from collecting ducts (RV.SVtsA58 cells and RC.SV3A2 cells) but not in cell lines derived from other segments of the nephron (RC.SV1 cells and RC.SV2 cells) or in fibroblastic cells (NIH/3T3 cells). The 7-bp mutation that abolished the enhancing activity of CSE1 in transfection experiments also abolished the binding of this cell-specific DNA-binding protein in gel shift assays.

The cell-specific DNA-binding protein binding to CSE1 has not yet been identified, but a computer-based analysis of CSE1 showed that it contains a potential binding site for MADS-box transcription factors related to MEF-2 and another one for winged-helix proteins, in addition to a consensus binding site for TBP (TATA binding protein) and to a weaker consensus binding site for the ubiquitously expressed transcription factor Oct-1 (14). DNA-binding proteins containing a MADS-box have been involved in cell differentiation and, for example, knock-out experiments have shown that MEF-2 plays an important role in muscle development (reviewed in Ref. 16), but no MEF-2-related protein is known to be present in the developing kidney. Furthermore, in gel shift experiments, MEF-2 and MEF-2-related proteins such as

MEF-2C give rise to very low-mobility complexes (17). Thus, it seems somehow unlikely that the cell-specific protein binding to CSE1 belongs to the MADS-box family. Forkhead/winged-helix proteins have also been implicated in cell fate determination and in cell-specific gene expression, and at least nine winged-helix transcription factors have been shown to be expressed in the developing kidney (18-26). Nevertheless, almost all of these transcription factors are expressed in the metanephric mesenchyme, and not in the ureteric bud or in its derivatives (18-24). Among forkhead transcription factors that are expressed in the ureteric bud, HNF-3 is expressed in the urothelium of the renal pelvis but not in collecting ducts (25), and similarly HFH-4 is expressed in the ureteric bud before its differentiation in collecting ducts but not in collecting duct cells (26). It is therefore possible that the cell-specific protein that binds to CSE1 is a yet unknown DNA-binding protein, and that it belongs to the winged-helix family of transcription factors. This hypothesis is supported by the recent identification of a cDNA encoding a winged-helix protein that is expressed selectively in collecting ducts cells and which starts to be expressed at a time corresponding to the expression of the V2 receptor (A. Calmont, unpublished results).

In transient transfection experiments, a construct containing 2,061 bp of the V2 receptor proximal promoter was at least 35-fold less active in all renal cell lines than a construct containing only 1,004 bp of the same promoter (compare pluc 2.0 with pluc 1.0), which suggests that a suppressor *cis*-acting element, named CIE1, is located between -2.0 kb and -1.0 kb. CIE1 may be important to silence transcription of the V2 receptor gene in cells that do not express the V2 receptor, in the same way as repressor elements present in the L1 adhesion molecule gene, in the Fgf4 gene, or in the GATA-1 gene (27-29). For each of these three genes, deletion of a repressor *cis*-acting element induced a ubiquitous expression of the reporter gene, in transgenic animals (27-29). In this model, CSE1 would act mostly by counteracting the inhibitory effects of CIE1. This hypothesis is supported by the results of transient transfection experiments performed using constructs containing or not containing CIE1. In collecting duct cell lines, a construct containing CSE1 cloned upstream of CIE1 was at least 30 times more active than a construct that did not contain CSE1 (compare pluc 2.24 with pluc 2.0). Conversely, a construct containing CSE1 cloned upstream of a 1-kb segment of the V2 receptor proximal promoter, and which did not include CIE1, was only about 3 times more active than a construct containing only the 1-kb promoter segment (compare pluc 1.0+ and pluc 1.0). Thus, our data suggest that CSE1 is a rather weak enhancer, but that it can very efficiently antagonize the inhibitory effects of CIE1 (Fig. 8).

In conclusion, we have identified a 33-bp segment of the V2 receptor promoter, named CSE1, which in-



**Fig. 8.** Model for the Action of the Cell-Specific Protein Binding to CSE1

A, Summary of the results of transfection experiments performed using constructs containing various combinations of CSE1, of CIE1, and of the V2 receptor minimal promoter (m.p.) (see *Results* for details). B, Schematic representation of the interactions between proteins binding to CSE1, to CIE1, and to the V2 receptor minimal promoter (m.p.). In this model, collecting duct cells contain a cell-specific protein able to bind to CSE1 and to inhibit the repressor effect of CIE1. This DNA-binding protein has also a weak enhancer activity on the V2 receptor minimal promoter (see text for details).

duced high levels of expression of the luciferase reporter gene specifically in collecting duct cell lines, and which bound a DNA-binding protein expressed selectively in these cell lines. CSE1 appears to act mostly by relieving the inhibitory effects of an ubiquitous repressor element named CIE1. CSE1 is the first functional collecting duct-specific *cis*-acting element identified so far.

## MATERIALS AND METHODS

### Cell Lines

Four rabbit cell lines derived from renal cortex and obtained in our laboratory were used in transfection experiments. The RC.SVtsA58 cell line has been obtained by infection of isolated renal cortical cells with the temperature-sensitive SV40

mutant tsA58, and its characteristics are those of principal cells of the collecting ducts (10–12). Run on experiments having shown that the V2 receptor gene is transcribed at similar levels at permissive temperature (33 C) and at restrictive temperature (39.5 C) (R. Piedagnel, unpublished results), all transfection experiments were performed at 33 C. The RC.SV1 cell line, the RC.SV2 cell line, and the RC.SV3 cell line have been obtained by infecting isolated renal cortical cells with wild-type SV40, and they were cultured at 37 C. The RC.SV3 cell line, and its subclone RC.SV3A2 cells, have functional characteristics of principal cells of the collecting ducts (9). RC.SV1 cells and RC.SV2 cells have characteristics of proximal tubular cells, and of cells of the thick ascending limb of the Henle's loop, respectively (9). All four rabbit cell lines were grown in 50% DMEM (Life Technologies, Inc., Gaithersburg, MD), 50% HAM (Life Technologies, Inc.) supplemented with 2 mM glutamine, 5 mg/liter insulin, 50 nM dexamethasone, 5 mg/liter transferrin, 30 nM selenium, and 20 mM HEPES (MD medium).

HCD.A8 cells, which are a subclone of the human HCD cell line (30), were provided by M. Géniteau-Legendre. The HCD

cell line has been obtained by transfection of isolated human renal cortical cells with a recombinant plasmid DNA harboring a complete but replication-defective SV40 genome (30). It has characteristics of principal cells of the collecting ducts, since it expresses the cell adhesion molecule L1 and responds to AVP by an increased production of cAMP (11, 30). These cells were grown in MD medium supplemented with 2% FCS (Life Technologies, Inc.).

NIH/3T3 mouse fibroblasts were obtained from ATCC (Manassas, VA), and cultured in DMEM supplemented with 10% FCS (Life Technologies, Inc.).

The Caco 2 cell line, which is derived from a human colon carcinoma, was given to us by G. Trugnan. It was grown in DMEM supplemented with 20% FCS (Life Technologies, Inc.) and 1% (vol/vol) nonessential amino acids.

### DNA Constructions

For transfection experiments, different inserts were cloned in the pluc 5 promoterless luciferase expression vector, which was obtained by modifying the polycloning site in pluc 4 (31). In this vector, the firefly luciferase gene is cloned upstream of an SV40 splice site and polyadenylation site and downstream of a polyadenylation cassette that prevents read-through transcription. Most inserts were derived from a cosmid named QC7C1, which contains an insert extending from about 19 kb upstream of the first exon of the V2 receptor to about 18 kb downstream of this gene (GenBank accession number U52111). In some constructs, a 47-bp fragment of the mouse  $\beta$ -globin proximal promoter was used as an heterologous minimal promoter, as in other studies (32).

To describe the promoter segments of the V2 receptor gene that we used, we numbered the nucleotides according to the transcription start site defined by RACE experiments (cf. *Results*). Nucleotide +1 is thus located 422 bp upstream of the translation initiation codon. pluc 2.0 contains a *Bam*HI-*Bam*HI segment of the V2 receptor gene extending from -2,061 bp to +192 bp, cloned upstream of the luciferase reporter gene in pluc 5. pluc 4.2 contains a -4,255-bp to +192-bp segment of the V2 receptor gene, cloned in pluc 5. It was generated by cloning a *Bgl*II-*Sac*II segment upstream of a -1,004-bp to +192-bp element derived from pluc 2.0. pluc 4.0 contains a -4,046-bp to +192-bp segment of the V2 receptor gene. It was derived from pluc 4.2 by deleting a *Bgl*II-*Hind*III fragment. pluc 2.4 was derived from pluc 4.2 by deleting an *Xba*I-*Nco*I fragment extending from -4,107 bp to -2,205 bp. pluc 2.24 was derived from pluc 2.0 by cloning a double-stranded oligonucleotide corresponding to the sequence extending from -4,140 bp to -4,107 bp (TACTC-TATGATTCTATTTATATAAAATTCTAGA) upstream of the *Nco*I site located at -2,205 bp. pluc 2.24m7 is identical to pluc 2.24 except that we used a double-stranded oligonucleotide containing a 7-bp mutation (TACTCTATGATTC-CCCCGGGGATAAAATTCTAGA, mutated nucleotides being underlined). pluc 0.05+ was derived from pluc 2.24 by deleting a *Nco*I-*Bgl*II fragment extending from -2,205 bp to -10 bp. pluc 1.0+ was derived from pluc 2.24 by deleting a *Nco*I-*Sac*II fragment extending from -2,205 bp to -1,004 bp. pluc 1.0, which contains a segment located between -1,004 bp and +192 bp, was derived from pluc 2.24 by deleting a *Bgl*II-*Sac*II fragment. pluc 0.7+ was derived from pluc 2.24 by deleting a *Nco*I-*Bst*XI fragment extending from -2,205 bp to -736 bp. pluc 0.4+ was derived from pluc 2.24 by deleting a *Nco*I-*Apal* fragment extending from -2,205 bp to -393 bp.

For transfection experiments, in addition to the constructs described above, we used: pluc 47G, which contains 47 bp of the mouse  $\beta$ -globin proximal promoter cloned in pluc 5; pSV- $\beta$ -galactosidase control vector (Promega Corp., Madison, WI), which contains the SV40 early promoter and enhancer cloned upstream of the *lacZ* gene; pSV Neo (Promega Corp.), which contains the SV40 early promoter and enhancer cloned upstream of the neomycin resistance gene; and pBS-

8M, which contains a 33-bp sequence extending from -4,140 bp to -4,107 bp multimerized eight times in pBlue-script KS (Stratagene, La Jolla, CA). Multimerization was made possible by introducing a *Bam*HI site at one end of the sequence and a *Bgl*II site at the other end.

### Transient Transfection Experiments

The day before transfection, cells were plated in six-well 25-mm dishes (Nunc, Kamstrup, Denmark) at a density of  $2 \times 10^5$  cells per well. Transfections were carried out with plasmid DNA coated with the polycationic lipid lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions. Briefly, 900 ng of a luciferase construct and 100 ng of pSV- $\beta$ -galactosidase control vector were mixed with 6  $\mu$ l of lipofectamine. Cells were incubated with this mixture in 1 ml serum-free medium for 24 h. They were then washed twice in PBS (150 mM NaCl, 10 mM sodium sulfate, pH 7.8) and incubated for 48 h in a medium containing twice the normal concentration of FCS. Cells were then washed twice with cold PBS, harvested on ice by scraping them in 0.2 ml potassium phosphate, 0.1 M, pH 7.8, and lysed by three freezing-thawing cycles.

Luciferase activity was assayed using a luminometer (EG&G Berthold, Bad Wilbad, Germany). Briefly, 50  $\mu$ l of cell lysates were incubated with 28 ng of D-luciferin (Fermentas AB, Vilnius, Lithuania) in 100 mM potassium phosphate, pH 7.8, 5 mM ATP, 15 mM MgSO<sub>4</sub>, and 1 mM dithiothreitol, and light units were counted for 5 sec (31).

$\beta$ -Galactosidase activity was used to correct for transfection efficiency. It was measured by a colorimetric assay using resorufin  $\beta$ -D-galactopyranoside (Sigma, St. Louis, MO) as a substrate, as previously described (33). Briefly, 20  $\mu$ l of cell lysates were incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 75  $\mu$ g resorufin  $\beta$ -D-galactopyranoside in dimethylsulfoxide), at 30 C. When a significant change in color was seen,  $\beta$ -galactosidase activities were measured at 572 nm.

All transfection experiments were done in triplicate and repeated at least three times. Results are expressed as mean  $\pm$  SD.

### Stable Transfection Experiments

Stable transfections were carried out as previously described for transient transfections with the following modifications. The day before transfection, cells were plated in 90-mm dishes (Nunc) at a density of  $10^5$  cells per dish. Linearized pluc 4.4 (4.5  $\mu$ g) and 0.5  $\mu$ g of linearized pSV neo were mixed with 30  $\mu$ l of lipofectamine. Transfected cells were selected in MD medium containing 100  $\mu$ g/ml of G418 (Sigma). Cells transfected in the same manner but without pSV Neo and cultured in the same selecting medium died after 1 week of culture.

### Determination of the Transcriptional Start Site

The transcriptional start site of the human V2 receptor gene was determined by using a 5'-RACE system (Life Technologies, Inc.) and RC.SV3A2 cells stably transfected with pluc 4.2. These cells harbor a transgene containing a segment of the V2 receptor gene extending from -4,255 bp to +192 bp, cloned upstream of the luciferase reporter gene. Briefly, total RNA was prepared according to Chomczynski and Sacchi (34), and used as a template to synthesize first-strand cDNA by reverse transcription in the presence of a luciferase-specific antisense primer (GSpluc1, 5'-AACACTACGGTAG-GCTGCGAAATG-3'). The 5'-end of this first-strand cDNA was labeled using dCTP and the terminal deoxynucleotidyl transferase. The tailed DNA was then used to perform the first PCR using a sense DNA primer complementary to the 5'-tail

and a second luciferase-specific antisense primer (GSPluc2, 5'-GCAACTCCGATAAATAACGCGCCC-3'). Samples were amplified for 45 cycles under the following conditions: denaturation for 60 sec at 94 C, annealing for 45 sec at 55 C, and extension for 90 sec at 72 C. Aliquots of the first PCR reaction were used as template in a second PCR. This nested PCR was performed using a nested sense DNA primer complementary to the 5'-tail, paired with a third luciferase-specific antisense primer (GSPluc3, 5'-CATAGCTTGTGCAACCGAACGGA-3'). Samples were amplified for 45 cycles under the following conditions: denaturation for 60 sec at 94 C, annealing for 45 sec at 55 C, and extension for 90 sec at 72 C. The PCR products were electrophoresed through a 1% agarose gel, excised, cloned into the pGEM-T-easy vector (Promega Corp.), and sequenced.

#### Decoy Experiments and Determination of cAMP Production

In a first set of experiments, RV.SV3A2 cells stably transfected with pluc 4.2 were transiently transfected with various amounts of either the decoy construct (pBS-8M) or pBlue-script KS. pSV- $\beta$ -galactosidase (0.1  $\mu$ g) was cotransfected to assess transfection efficiency. Luciferase and  $\beta$ -galactosidase activities were assayed as described above.

In a second set of experiments, RC.SVtsA58 cells, cultured in six-well dishes at 33 C, were transfected with various amounts of either the decoy construct (pBS-8M) or pBlue-script KS. After 3 h, the cells were switched to 39.5 C, to allow the expression of V2 receptors at the cell surface. After 36 h, determination of cAMP production in response to AVP was performed as previously described (9). Briefly, cells were first incubated for 10 min at 37 C in MD medium containing 0.1 mM 3-isobutyl 1-methyl xanthine (IBMX, Sigma). Thereafter, they were incubated for 7 min at 37 C in the same medium supplemented or not with  $10^{-7}$  M AVP (Sigma) or  $10^{-6}$  M forskolin (Sigma). The reaction was stopped by rapid removal of the medium, immediately followed by addition of 1 ml of a solution containing 95% ice-cold ethanol and 5% formic acid. After 45 min of incubation at 4 C, the supernatant was recovered, evaporated, and resuspended in 50 mM sodium acetate, pH 6.2, and used in a RIA. cAMP production was calculated as picomoles per  $\mu$ g of protein. To measure the protein content of each cell culture, 1 ml of 1M NaOH was added to each well. After overnight incubation at 4 C, supernatants were removed and protein content was measured according to Bradford (35).

#### Gel Retardation Assays

Nuclear extracts were prepared from 80% confluent cells as previously described (36), except that the buffer used to extract nuclear proteins contained 0.55 M NaCl. Fifteen femtomoles of a double-stranded oligonucleotide probe labeled with the Klenow fragment of *Escherichia coli* DNA polymerase I and 30  $\mu$ Ci [ $\alpha$ - $^{32}$ P] dCTP were incubated for 30 min at room temperature in the presence of 5–8 mg of nuclear proteins, 300 ng of poly(dI-dC):poly(dI-dC) (Amersham Pharmacia Biotech, Björkgatan, Sweden), and 1  $\mu$ g of salmon sperm DNA (Roche Molecular Biochemicals, Indianapolis, IN), in a solution containing 20 mM HEPES, pH 7.9, 25 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% Nonidet P-40, and 8% glycerol. The reaction mixture was then fractionated by electrophoresis on a 4% polyacrylamide gel. Competition experiments were performed in the presence of a 50- to 100-fold molar excess of competitor.

#### Acknowledgments

We thank B. de Crombrughe and G. Trugnan for the generous gift of the mouse  $\beta$ -globin minimal promoter and of the

Caco 2 cell line, respectively. We thank M. Géniteau-Legendre for providing us with HCD.A8 cells. We thank J. Chambaz, C. Terraz, and R. Piedagnel for carefully reading the manuscript.

Received February 14, 2000. Revision received June 22, 2000. Accepted July 3, 2000.

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This work was supported by Grant 9871 from the Association pour la Recherche sur le Cancer (to J. R.), by INSERM, and by The University of Paris VI. A. C. is a recipient of a fellowship from the Ministère de l'Education Nationale.

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