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Negative control elements and cAMP responsive sequences in the tissue-specific expression of mouse renin genes

(gene regulation)

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ABSTRACT **The 5′ flanking regions of the mouse renin genes (*Ren1^d^* and *Ren2^d^*) contain putative negative control and cAMP responsive elements. Sequence analysis shows additionally that these putative control elements in the *Ren2^d^* gene are interrupted by a 160-base-pair insertion. To document the functions of these elements, we isolated these regions and fused them to the reporter gene chloramphenicol acetyltransferase (CAT), which was linked upstream to a thymidine kinase (TK) promoter (pUTKAT1). The chimeric constructs were transfected into mouse pituitary tumor AtT-20 and human choriocarcinoma JEG-3 cells. At the basal unstimulated condition, *Ren1^d^* 5′ flanking sequence in the sense orientation inhibited basal CAT expression from the TK promoter of pUTKAT1, whereas the same sequence in the antisense orientation did not. The 5′ flanking region of *Ren2^d^* had no inhibitory effect on basal CAT expression. These data demonstrate that the negative control element is functional in *Ren1^d^* but is nonfunctional in *Ren2^d^*, suggesting that the 160-base-pair insertion in *Ren2^d^* interferes with the function of the negative control elements. In response to 8-bromo-cAMP, both renin genes increased transcription 3-fold, suggesting a functional cis action of the cAMP responsive element in both genes. These data may be important in the understanding of the regulation of the tissue-specific expression of mouse renin genes.**

Circulating renin is mainly synthesized in the juxtaglomerular cells of the kidney and participates in the generation of a potent vasopressor peptide, angiotensin II. In some strains of mice, the submandibular gland (SMG) also synthesizes high amounts of renin (1). Mouse strains with high levels of SMG renin, such as DBA/2J, carry two renin genes (*Ren1^d^* and *Ren2^d^*), whereas mouse strains with low levels of SMG renin, such as C57BL/10, carry a single renin gene (*Ren1^c^*) (2). *Ren1* is expressed mainly in the kidney, whereas *Ren2^d^* is expressed in both kidney and SMG (3). Sodium depletion increases renin mRNA in the kidney but not in the SMG (4). On the other hand, SMG renin, in contrast to kidney renin, is influenced by androgen (5). These data demonstrate that renin expression is regulated in a tissue-specific manner, but it is unclear whether this regulation is due to a transcriptional regulation of the renin genes (*Ren1^d^* and *Ren2^d^*).

Recently, we determined the entire nucleotide sequence of the *Ren1^d^* gene and compared the 5′ flanking regions of *Ren1^d^* and *Ren2^d^* genes (unpublished data). Sequence analysis suggests that the 5′ sequences of both genes are highly homologous. The *Ren1^d^* gene contains a putative cAMP responsive element. In the *Ren2^d^* gene, this sequence is interrupted by a 160-base-pair (bp) sequence (Fig. 1) whose functional significance is unclear. Furthermore, sequences homologous to the negative control elements of the chicken lysozyme gene (6) are also identified in both *Ren1^d^* and *Ren2^d^* genes. It is unknown whether the 160-bp insertion present in the *Ren2^d^* gene interferes functionally with the putative control elements.

In this study, chimeric constructs of the 5′ flanking region containing the putative negative and cAMP responsive elements from *Ren1^d^* and *Ren2^d^* genes, a truncated thymidine kinase (TK) promoter, and the reporter gene encoding chloramphenicol acetyltransferase (CAT) were used for studies of transient expression to document the effects of these putative elements on gene transcription from the TK promoter. Our results demonstrate that both *Ren1^d^* and *Ren2^d^* genes conserve cAMP-inducible enhancer element, which is functional irrespective of the presence or absence of the 160-bp insertional element. On the other hand, this 160-bp element influences the function of the negative control element, which becomes inactive in the presence of this insertional element (i.e., *Ren2^d^* gene) but is active in the absence of the element (i.e., *Ren1^d^* gene).

MATERIALS AND METHODS

Cell Lines. Mouse pituitary tumor (AtT-20) and human choriocarcinoma (JEG-3) cell lines were obtained from the American Type Culture Collection and were grown in media as recommended by the supplier. These cells were selected for use because of their previously documented ability to respond to cAMP (7, 8).

Plasmid Constructs. The 5′ flanking regions of *Ren1^d^* and *Ren2^d^* genes were isolated from the plasmid subclones, pRn34 and pBa6, respectively. pRn34 is a 6.5-kilobase (kb) *Bam*HI fragment from the *Ren1^d^* gene containing the 5′ flanking region, exon 1, and part of the first intron, inserted into the *Bam*HI site of pUC8. This *Bam*HI λ fragment was originally isolated from a λ DBA renin genomic clone, DBARn12, containing the *Ren1^d^* gene (9). pBa6 contains a 5-kb *Bam*HI fragment from the 5′ flanking region of the *Ren2^d^* gene inserted into the *Bam*HI site of pUC8. This *Bam*HI fragment was originally isolated from a λ DBA renin genomic clone λ2-12 containing the *Ren2^d^* gene (provided by K. W. Gross, Roswell Park Memorial Institute, Buffalo, NY).

Ren1 *pUTKAT1*. The 340-bp *Xba* I fragment from pRn34 containing the putative cAMP responsive and negative elements was cloned in either orientation into the *Xba* I site of pUTKAT1, a CAT expression vector that contained a truncated TK promoter (10).

Ren2 *pUTKAT1*. The 480-bp *Xba* I fragment from pBa6 containing the putative cAMP responsive and negative ele-

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Abbreviations: SMG, submandibular gland; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase.

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ments was cloned in either orientation into the *Xba* I site of pUTKAT1.

Transfection. Cells were plated at 10^6 cells per 100-mm dish 1 day prior to transfection. Plasmid DNA (10 μg) purified on a cesium chloride gradient was transfected into cells by the calcium phosphate method described by Gorman (11). Cells were exposed to calcium phosphate/DNA precipitate for 4 hr at 37°C followed by 2 min of glycerol shock and were reincubated for 24 hr at 37°C. The following day, cells were further incubated for 20 hr at 37°C in the absence or presence of 2 mM 8-bromo-cAMP (Sigma) before assaying for CAT activity.

CAT Assay. Cells were harvested with trypsin/EDTA (GIBCO), washed with cold phosphate-buffered saline, and lysed in 100 μl of 0.25 M Tris·HCl, pH 7.5/5 mM EDTA/0.1% Triton X-100. After centrifugation in a Microfuge for 5 min at 4°C, the supernatant was treated at 60°C for 10 min to inactivate endogenous acetylases (12) and the final supernatant was assayed for CAT activity. Protein concentration was determined by the Bradford method (13) (Bio-Rad). CAT activity was measured as described by Gorman *et al.* (14). Identical amounts of protein per sample (50–100 μg) were incubated with 40 mM acetyl CoA (Pharmacia P-L Biochemicals), 1% glycerol, and 0.2 μCi of [^{14}C]chloramphenicol (1 Ci = 37 GBq) (New England Nuclear) for 16 hr at 37°C.

Statistical Analysis. The experiment was repeated five times in mouse AtT-20 cells and four times in JEG-3 cells.

Values are given as means ± SEM, and statistical analysis was by the Student's *t* test.

RESULTS

Sequence Analysis of 5′ Flanking Regions of Mouse Renin Genes. As shown in Fig. 1, the composite physical maps for *Ren1^d* and *Ren2^d* genes show a high degree of homology in the 5′ flanking region; however, the *Ren2^d* gene has two additional insertions. The first insertion is a B2 repeat element and the other is a 160-bp insertion. These are located 210 and 615 bp upstream from the start of transcription, respectively. Both genes contain a 29-nucleotide sequence that has a high degree of homology to the cAMP responsive sequence reported by Nagamine and Reich (15) (see *Discussion*). This sequence is conserved in a number of cAMP-inducible genes.

cAMP Responsive Element. We subcloned a 340-bp fragment from *Ren1^d* and a 480-bp fragment from the *Ren2^d* gene into the *Xba* I site of pUTKAT1 to determine whether these sequences were sufficient to induce a cAMP response (Fig. 1). pUTKAT1 was used as a control. Constructs in either orientation were transfected into mouse AtT-20 cells. When the transfected cells were treated with 2 mM 8-bromo-cAMP for 20 hr, CAT activities in both *Ren1^d* pUTKAT1 and *Ren2^d* pUTKAT1 increased ≈3-fold ($P < 0.005$). This positive response was seen for both constructs independent of orientation of the fragment (Fig. 2*A*). In contrast, 8-bromo-cAMP had no effect on pUTKAT1 expression itself. pRSV–CAT containing Rous sarcoma virus promoter linked to the CAT gene (11) was also used as a control for nonspecific effects of cAMP on gene expression. No induction of CAT activity by 8-bromo-cAMP was observed with pRSV–CAT (data not shown). The overall result was similar to that observed by Comb *et al.* (16) after transfection into CV-1 cells of a chimeric construct consisting of the 5′ flanking region of the proenkephalin gene and pUTKAT3, a derivative of pUTKAT1. These results indicate that the effect of cAMP is specific and is dependent on the presence of cAMP responsive element in a mouse renin gene fragment derived from either the *Ren1^d* or *Ren2^d* genes.

Negative Control Element. *Ren1^d* (340 bp) and *Ren2^d* (480 bp) gene fragments containing regions of homology with the negative control element of the chicken lysozyme gene (6)

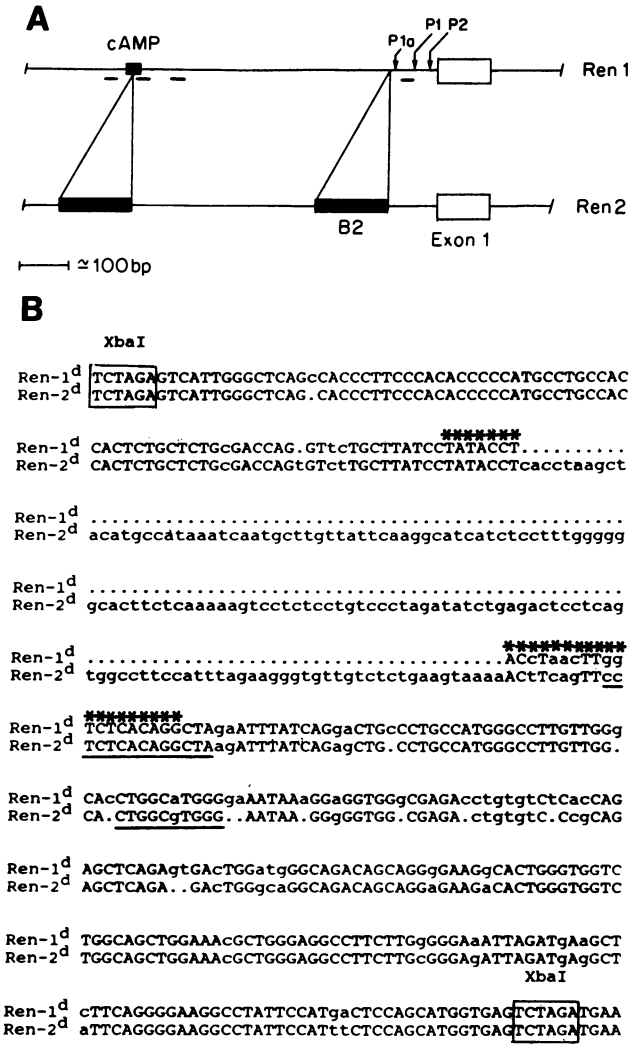

FIG. 1. (*A*) Alignment of 5′ flanking regions of mouse *Ren1^d* and *Ren2^d* genes. Sequence analysis shows a high degree of homology. Renin promoters are named P1a, P1, and P2. Insertions of a B2 repeat element and a 160-bp element in the *Ren2^d* gene are represented schematically. Sequences homologous to the negative control element of chicken lysozyme gene are underlined. (*B*) Sequence of the putative cAMP responsive regions of mouse renin genes. The cAMP responsive element is denoted by asterisks and the negative element is underlined.

were studied (Fig. 1). The basal CAT activity of each construct was compared to that of pUTKAT1 (Fig. 3*A*). *Ren1^d* pUTKAT1 in the antisense orientation and *Ren2^d* pUTKAT1 in both orientations showed no effect on the expression of CAT in the absence of cAMP. In contrast, *Ren1^d* pUTKAT1 in the sense orientation repressed CAT expression by >60% ($P < 0.005$).

Transfection of Mouse Renin–CAT Fusion Genes into Heterologous Cell Lines. It has been reported that human chorionic cells synthesize renin (17). Recently, we demonstrated that the human renin promoter is functional in JEG-3 cells (18). To test whether the cAMP-induced expression of the mouse renin–CAT fusion gene is cell specific (AtT-20) or can be extended to other renin-producing cell types besides the AtT-20 cell, 5′ mouse renin–pUTKAT1 fusions were transfected into JEG-3 cells. The treatment of cultures with 2 mM 8-bromo-cAMP resulted in a 3-fold increase of CAT activity in each construct ($P < 0.05$) (Fig. 2*B*). Furthermore, *Ren1^d* pUTKAT1 in the sense orientation showed repression of CAT activity similar to that found in mouse AtT-20 cells (Fig. 3*B*).

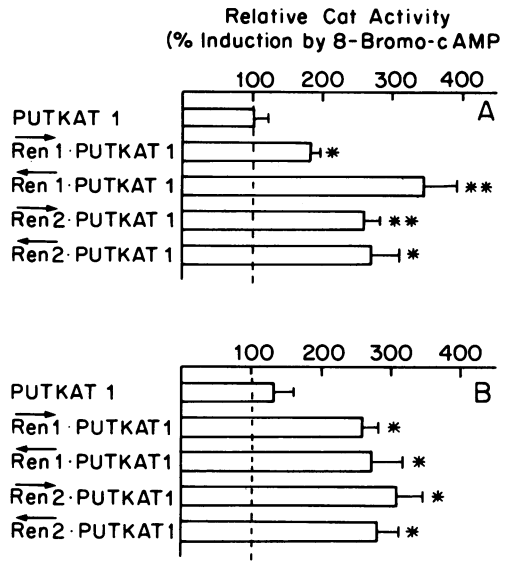

FIG. 2. Effect of 8-bromo-cAMP on CAT activity in transfected mouse AtT-20 (*A*) and JEG-3 (*B*) cells. The relative CAT activity of each construct was calculated by comparing the percentage conversion of [^{14}C]chloramphenicol to its acetylated forms by the 8-bromo-cAMP-treated samples to the untreated controls. *, $P < 0.05$; **, $P < 0.005$ compared to pUTKAT1.

DISCUSSION

cAMP Responsive Element. Our studies demonstrate that the 5′ flanking sequences of *Ren1^d* and *Ren2^d* genes contain elements that confer cAMP responsiveness on the truncated TK promoter, whose function by itself is not regulated by cAMP. Indeed, a potential cAMP responsive sequence, which is >45% homologous to the sequence reported by Nagamine and Reich (15), is found in the mouse renin genes. This sequence contains 29 nucleotides that are homologous to the sequences present in porcine uPA (19), rat tyrosine aminotransferase (20), and rat prolactin (21) genes, which are all regulated by cAMP.

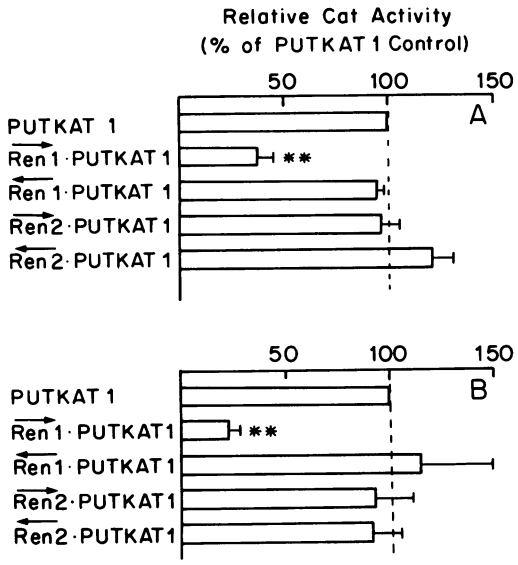

FIG. 3. Effects of mouse renin–pUTKAT1 fusion genes on basal unstimulated CAT activity in transfected mouse AtT-20 (*A*) and JEG-3 (*B*) cells. The relative CAT activity was calculated by dividing the basal CAT activity of each construct by that of pUTKAT1. Results are expressed as percentage of the pUTKAT1 control. **, $P < 0.005$ compared to pUTKAT1.

It is surprising that a putative cAMP responsive element of the *Ren2^d* gene is still able to respond to cAMP despite the physical interruption by a 160-bp insertion. Montminy *et al.* (22) reported that the core sequence is a component of the cAMP regulatory region of the rat somatostatin gene, but it is not in itself sufficient for cAMP responsiveness. This observation was also found in the rat phospho*enol*pyruvate carboxykinase gene (23). Therefore, it is unlikely that the 160-bp insertion in the *Ren2^d* gene mediates/modulates cAMP inducibility but the flanking sequence adjacent to the cAMP responsive element may be important for cAMP inducibility. It is unknown whether this 160-bp sequence represents a newly discovered repetitive sequence. A search did not reveal any significant homologies with other sequences in the May 2, 1986, Release 42.0 of the GenBank data base.

Regulation of renin secretion is well established. In the mouse, β_1-adrenoreceptor stimulates renin secretion from the kidney via the cAMP-dependent pathway but not from the SMG (24). On the other hand, renin secretion from the SMG is stimulated by α_1-adrenoreceptor. Our recent study showed that stably transfected mouse AtT-20 cells with mouse (*Ren2^d*) cDNA secreted mature one-chain renin, and this one-chain renin secretion was stimulated by 8-bromo-cAMP (25). Isoproterenol treatment, a β-adrenergic agonist, also increases kidney renin mRNA in mice (26), suggesting that cAMP is able to increase transcription of the renin gene *in vivo*. Taken together, our studies indicate clearly that *Ren1^d* and *Ren2^d* genes conserve a cis-acting sequence for cAMP responsiveness. The nonresponsiveness of *Ren2^d* secretion to isoproterenol in the SMG must therefore be attributed to tissue-specific events—e.g., trans-acting factors—or synthesis–secretion coupling.

Negative Control Elements. Transcription of eukaryotic genes is regulated by different tissue-specific factors. It is proposed that some genes contain negative regulatory elements that can inhibit the transcription of these genes under certain conditions (27). In the present study, a *Ren1^d* 5′ flanking fragment in the sense orientation represses basal CAT activity, suggesting the existence of cis-acting negative control element(s). Indeed, this 5′ flanking fragment contains two sequences homologous to the negative elements of chicken lysozyme gene (6). The first sequence is located at the 3′ end of the putative cAMP response sequence and the second sequence is located ≈50 bp downstream of it. These sequences are functional in only one orientation and are not, by definition, silencer sequences as observed in other genes (6, 28). Furthermore, they do not inhibit cAMP responsiveness. Therefore, the negative control elements in *Ren1^d* may be responsible for modulating basal levels of renin gene expression in the absence of other regulatory factors. In contrast, *Ren2^d* 5′ flanking sequences do not repress basal CAT activity. Thus, the presence of the 160-bp insertion sequence interferes with the function of the *Ren2^d* "inhibitory" sequences. Our observations that the negative control elements are functional in *Ren1^d* but not in *Ren2^d* genes support the hypothesis of Field and Gross (3). These investigators postulated that the reduced level of *Ren1^d* gene expression in the SMG (by a factor of 100) relative to *Ren2^d* expression in the SMG was due to nonresponsiveness of the *Ren2^d* gene to a negative control. In other words, in the SMG, the *Ren1^d* gene is silent in the basal state in SMG through the influence of a negative element. However, it remains to be tested whether an identical 160-bp insertion in *Ren1^d* will inhibit the action of the negative control element.

In conclusion, we have demonstrated that both *Ren1^d* and *Ren2^d* genes conserve functional cis-acting cAMP responsive elements. We report herein a negative control element in the *Ren1^d* gene, which is nonfunctional in the *Ren2^d* gene because of the presence of a 160-bp sequence. The identifi-

cation of the negative control elements in the mouse renin gene provides insight into the tissue-specific regulation of renin gene expression.

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