# The activity of the mouse renin promoter in cells that do not normally produce renin is dependent upon the presence of a functional enhancer

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The expression of a hybrid gene containing the promoter region of the mouse *Ren-1* or *Ren-2* genes and the chloramphenicol acetyl transferase (CAT) gene coding region was analysed in five cell lines that do not normally express the renin gene. The renin promoter is inactive in each of these cell lines unless the SV40 enhancer is also present in the construct. In the latter case, transcription initiates at the normal renin start site. This is in contrast to the situation observed in lymphoid cells where the renin promoter is inactive even when coupled to a functional enhancer [(1987) EMBO J. 6, 1685–1690]. Furthermore, 5'-flanking sequences of *Ren-2*, placed upstream of the thymidine kinase or SV40 early region promoters do not alter the activity of these promoters in three different cell lines. The results suggest that, except for B cells, the renin promoter is not tissue-specific but that its lack of activity in cells that do not express the gene is not the result of repression but may be due to the absence in these cells of one or several *trans*-acting positive factors.

Renin; Submaxillary gland; Gene expression

## 1. INTRODUCTION

Promoters and enhancers constitute the two main classes into which the *cis*-acting elements responsible for the specific expression of a eucaryotic gene in a given tissue, at a specific stage of development or in response to a given stimulus can be divided. The promoter is responsible for accurate initiation of transcription whereas the enhancer increases the rate of transcription from a promoter which can be located as far as several kilobase pairs away. More recently, interactions of proteins with these *cis*-acting DNA sequences have been described and some of the protein factors characterized. An important issue that remains to be addressed is how the different DNA elements and/or DNA-protein complexes that are involved in the specific expression of a gene interact with one another.

The two renin genes of the mouse are particularly interesting in this respect. Designated by *Ren-1* and *Ren-2*, they are both expressed mainly in the juxtaglomerular cells of the kidney and in the submaxillary gland (SMG) although lower expression in other extra-renal sites (testis, adrenals, ovaries, brain) has been reported [1–3]. *Ren-1* and *Ren-2* are under the control of androgens and thyroxin in the SMG and *Ren-2* which is present in only certain strains of mice is expressed at levels higher than those of *Ren-1* by about two orders of magnitude in the SMG of both males and females. The proximal 5'-flanking regions of both genes have been

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sequenced [4,5] and comparison with the equivalent region of the unique renin gene of the rat, which is expressed only in the kidney, has yielded clues on the elements responsible for SMG expression and regulation by androgens [6]. Moreover, essentially all the information necessary for proper expression of Ren-2 is contained within a 16 kb fragment that includes 2.5 kb of 5'-flanking sequence, the entire coding region and 3 kb of 3'-flanking sequences, as demonstrated by experiments with transgenic mice carried out in this laboratory [7]. Unfortunately, the absence of cell lines that normally express the renin gene has hindered the detailed analysis of the cis-acting elements involved in the differential expression and hormonal regulation of Ren-1 and Ren-2. The present work is an effort to identify some of these elements using cell lines where renin expression is not spontaneously observed. It was previously demonstrated in this laboratory that the renin promoter is not functional in B lymphocytes even when coupled to an enhancer functional in these cells [8]. In the present work, we show that, in contrast to what is seen in B cells, the renin promoter is functional in five other cell lines, provided that an enhancer functional in the cell line is also included in the transfected construct indicating that the non-activity of the renin promoter alone is not due to the presence of a repressor.

## 2. MATERIALS AND METHODS

### 2.1. Plasmid construction

The structure of the plasmids used in this work is partially schematized in fig.1. The R2 fragment was obtained from the recombinant λ phage SW 10 [4]. It consists of positions −2500 to +7 (for numberings, see [9]) of Ren-2. It also carries positions 5171-5235 of the SV40 virus immediately downstream of the renin sequences (fig.3). The R1 fragment and its deletions are described in [8]. Both R2 and R1 fragments were subcloned into the unique restriction sites of pSB1 or pSB1SV [10] to generate pR2CAT, pR2CATSV and pR1CATSV. The pSB1SV plasmid which contains the SV40 enhancer (PvuII-NcoI [11]) in the unique BamHI site was used to make the negative control plasmid and pSB1 containing the SV40 early region promoter (the 342-bp PvuII-HindIII fragment corresponding to positions 270-5171) subcloned in the HindIII site was used to make the positive control. The 1025-bp UMS fragment (SacI-XbaI [12]) was kindly provided by P. Herbomel with SalI and XhoI sites at its respective ends. It was subcloned into the SalI site of the above CAT plasmids to generate ptR2CAT, ptR2CATSV, ptR1CATSV, the negative control plasmid (ptCATSV) and the positive control plasmid (ptSVECAT). The plasmid ptΔ5CATSV which constitutes a deletion mutant of

prR1CATSV was constructed from the R5 fragment described previously [8].

For the construction of plasmids containing the herpes simplex virus thymidine kinase (tk) promoter, the HindIII-BamHI fragment of pTKCAT [11] was isolated and ligated to the BamHI-HindIII fragment of ptCATSV to generate pttkCAT. BamHI linkers were ligated to the HindIII end of the same fragment of pTKCAT, followed by ligation to the BamHI fragment of ptR2CAT that contains the plasmid sequences, the UMS and R2 positions −2500 to −117 thus generating ptR2tkCAT (fig.1).

The plasmid ptR2SVECAT was constructed by subcloning the HindIII fragment containing the SV40 early promoter of ptSVECAT into the unique HindIII site of ptR2CAT and the plasmid ptR2tSVECAT was constructed by excision of the SalI-HindIII fragment of ptR2CAT (UMS and R2 sequences), addition of SalI linkers to the HindIII end of this fragment and insertion into the SalI site of ptSVECAT.

### 2.2. Cell culture, transfection and CAT assay

Baby hamster kidney (BHK), mouse L, and human choriocarcinoma JEG-3 cell lines were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Chinese hamster ovary (CHO) cells were grown in F12 medium supplemented with 10% fetal calf serum and cells of the human mammary tumor cell line T47D were grown in RPMI 1640 medium with 10% fetal calf serum. About $7 \times 10^5$ cells were plated in 10 cm Petri dishes 24 h before transfection. All cells were transfected using the calcium phosphate procedure [13], except for T47D cells which were transfected by the DEAE-dextran procedure [14] or by electroporation at 210 V (3.8 mm electrode spacing) [15]. For CAT assays, cells were suspended in 0.25 M Tris-HCl, pH 7.8, and lysed by successive cycles of freezing and thawing. CAT activity was assayed on 0.2-0.3 mg of cytosolic protein as described [16].

### 2.3. RNA preparation and primer extension analysis

BHK cells were transfected with 10 μg of plasmid. They were collected 48 h later, rinsed with ice-cold phosphate-buffered saline and lysed on plates with 4 ml of: 4 M guanidinium thiocyanate; 1 M β-mercaptoethanol; 20 mM sodium acetate, pH 5. RNA was prepared by guanidinium isothiocyanate/lithium chloride precipitation followed by oligo (dT)-Sepharose chromatography as in [17].

A single strand probe corresponding to positions 1-71 of the cat gene (downstream from the HindIII site) was prepared according to the procedure previously described [8]. The poly(A)+ RNA was hybridized to 25 000 cpm of the probe for 18 h at 42°C in 10 μl of hybridization buffer: 40 mM Pipes, pH 6.4; 1 mM EDTA; 0.5 M NaCl and 50% formamide. Following precipitation, the hybrids were elongated with reverse transcriptase (Genofit) and analysed as described by [9] except that the 4 dNTPs were present in the reaction mixture and that elongated hybrids were desalted by three successive steps of ethanol precipitation before being loaded onto a 6% sequencing gel.

## 3. RESULTS

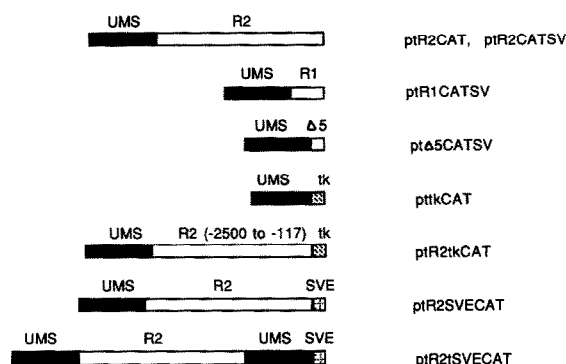Constructs containing 5'-flanking sequences from Ren-1 or Ren-2 (fig.1) linked to the indicator

Fig.1. Structure of the promoter fragments for the renin-CAT constructs. The above DNA fragments were subcloned into the SalI-HindIII cloning sites of pSB1 or pSB1SV [10], immediately upstream of the *cat* coding sequences. UMS, upstream mouse sequence; R2, 2.5 kb of 5'-flanking sequence of *Ren-2*; R1, 0.5 kb of 5'-flanking sequence of *Ren-1*; Δ5, a deletion mutant of R1 conserving positions − 142 to + 30; tk, herpes simplex virus thymidine kinase promoter positions − 200 to + 50; SVE, SV40 early promoter positions 270 to 5171 (342 nt). For details of the constructions, see section 2.



Fig.2. Expression of renin-CAT constructs in T47D cells. CAT activity was determined in extracts of T47D cells transfected 65 h earlier with 20 μg of the indicated plasmids by electroporation. Each sample corresponds to 300 μg of cytosolic proteins.

gene CAT were transfected into five different cell lines that do not normally produce renin: baby hamster kidney (BHK) and L fibroblasts, Chinese hamster ovary (CHO) cells, the JEG-3 choriocarcinoma cell line and the human mammary tumor cell line T47D. Some of the constructs also contained the SV40 enahncer [18]. In transient expression

Table 1

Expression of renin-CAT hybrid genes in heterologous cell lines

| Plasmid | Cell line | | | | |
|---|---|---|---|---|---|
| | L | CHO | BHK | JEG-3 | T47D |
| ptR2CAT | 0 | 0 | 0 | 0 | 0 |
| ptR2CATSV | 42 (0.8) | 27 (0.9) | 9 (0.4) | 0.6 (0.3) | 10 (1.3) |
| ptR1CATSV | 21 (0.4) | 27 (0.9) | 4 (0.2) | 0.2 (0.1) | 1 (0.1) |
| ptΔ5CATSV | ND | 9 (0.3) | ND | ND | ND |

CAT activity was determined on 0.2–0.3 mg of cytosolic protein 65 h after transfection. Numbers represent CAT activity expressed as the percentage of the positive control plasmid (ptSVECAT). The absolute CAT activity expressed as the % of acetylated chloramphenicol during a 2-h incubation is given in parentheses. All values are the mean of two to four experiments. ND, not determined
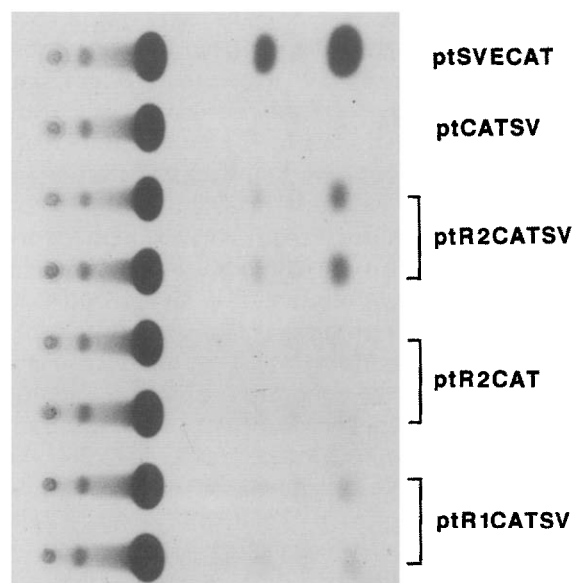
experiments, no CAT activity could be observed with the plasmids that only contained 2.5 kb of *Ren-2* 5'-flanking sequence. However, constructs that contained both the SV40 enhancer and renin sequences (2.5 kb of *Ren-2* or 0.5 kb of *Ren-1*) were active in each of the five cell lines that were tested (fig.2, table 1). The activity observed with the different plasmids expressed as a percentage of the positive control (ptSVECAT) varied according to the cell line but the absolute activity was always of the same order of magnitude. In four of the five cell lines, CAT activity obtained with ptR2CATSV was not significantly different from that obtained with ptR1CATSV. Only in T47D cells did the latter plasmid produce 10 times less activity than ptR2CATSV (table 1). In order to verify that the absence of CAT activity observed after transfection of ptR2CAT is not due to the absence from this construct of a crucial *cis*-acting element located elsewhere in the gene (introns, 3'-flanking region), a plasmid carrying the entire *Ren-2* gene (16 kb fragment; see section 1 and [8]) was transfected into T47D cells by electroporation and renin transcripts were tentatively detected 48 h later using the extremely sensitive cDNA amplification

procedure (Ekker et al., Proc. Natl. Acad. Sci. USA, in press) which derives from the polymerase chain reaction assay [19]. However, we could not detect any signal corresponding to a renin transcript (not shown). Therefore, even the entire renin gene seems to be inactive following transfection into T47D cells.

All hybrid constructs that were used in this work contain the UMS (upstream mouse sequence) element immediately upstream of the renin sequences (fig.1) or at the equivalent position in positive and negative control plasmids. The UMS sequence originates from the upstream region of the mouse c-*mos* gene and has been shown to be a potent transcription terminator in eucaryotic cells [12,20]. We have inserted this element in order to prevent back-

ground CAT activity resulting from spurious transcripts originating in the procaryotic sequences of the vector [21]. Previous observations in this laboratory showed that plasmids containing only renin sequences (the same as those shown in fig.1) but without the SV40 enhancer nor the UMS had lower activities than the easily detectable activity of the negative control and that these activities increased when the renin sequences were progressively deleted (unpublished observations). Such results could suggest the presence of a repressor-like element within the renin sequences. However, in this work, the negative control plasmid with UMS never produced any background CAT activity and reduction of the size of the renin sequences down to 180 bp did not lead to a significant enhancement of
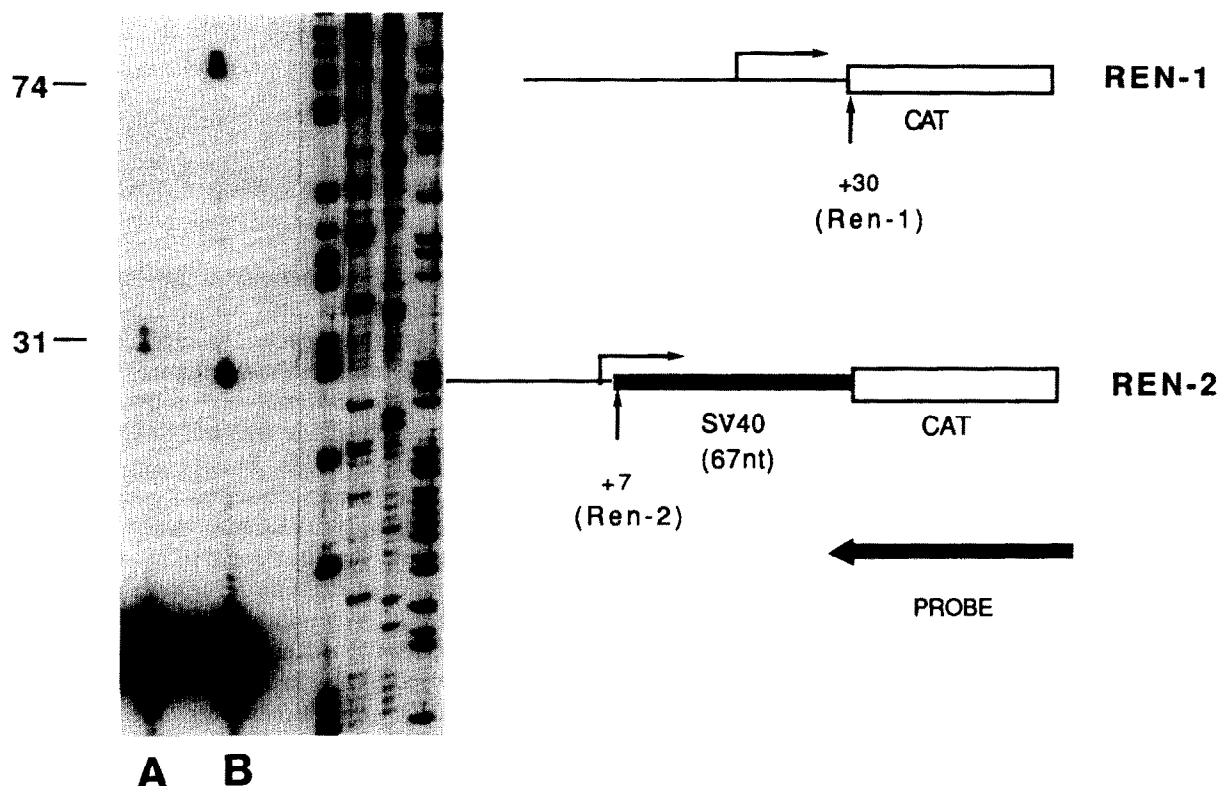


Fig.3. Mapping of the transcription startpoints for ptR1CATSV (lane A) and ptR2CATSV (lane B) in BHK cells. RNA was prepared from cells 48 h after transfection. Primer extension analysis was performed as described in section 2. The primer extension strategy is depicted on the right. Thin line: renin sequences with their 3'-end indicated by vertical arrows. Horizontal arrows indicate the start site and direction of transcription. The open box represents the *cat* coding region and the primer corresponds to *cat* positions 1–71 with respect to the *Hind*III site. In the *Ren*-2 construct, the dark box represents SV40 positions 5171 to 5235 + linker. The presence of these sequences is the result of the strategy used to prepare the R2 fragment. There is no ATG codon in the viral sequences and these, which are also found in the positive control plasmid, do not interfere with CAT activity. However, the spot around +29 in lane B is related to the presence of the SV40 sequences. The same spot is observed when the transcription startpoint is mapped for the positive control plasmid that contains the same sequences. It represents none of the SV40 early promoter start sites.

CAT activity as shown in table 1. Therefore, our previous observations can be best explained by non-specific initiation of transcription in the pBR322 sequences upstream of the renin fragments with decreased translatability into CAT protein of readthrough transcripts upon the insertion of long renin sequences.

We determined the site of transcription initiation of the renin-CAT fusion genes (ptR2CATSV and ptR1CATSV) in BHK cells by primer extension analysis with an oligonucleotide that corresponds to the 5'-end of the CAT gene coding region. Fig.3 shows that the length of the extension products that were obtained indicating that initiation of transcription occurred at the normal startpoints of Ren-2 and Ren-1, respectively. Therefore, The Ren-1 and Ren-2 fragments that we have used do function as promoters in BHK cells provided that the SV40 enhancer is also present in the construct. We assume that this is also the case for CHO, L, JEG-3 and T47D cells.

In order to identify elements within the 5'-flanking region of the mouse renin genes which have stimulatory effects on transcription, we placed *Ren*-2 sequences upstream of two well-characterized promoters that are known to function in a wide variety of cell types. In a first series of experiments, we replaced nucleotides −1 to −117 of ptR2CAT (with respect to the renin transcription start of Ren-2) by the well-characterized thymidine kinase promoter of herpes simplex virus [23]. This plasmid (ptR2tkCAT) as well as the equivalent plasmid without renin sequences (pttkCAT) were transfected into T47D cells or into rat pituitary GC cells. No difference in CAT activity could be observed between pttkCAT and ptR2tkCAT (table 2). Moreover, in GC cells in which regulation of growth hormone promoter activity by thyroid hormone has been demonstrated, the addition of 2.4 kb of renin sequences upstream of the tk promoter had no effect on the activity of the latter when the cells were grown in the presence of $10^{-9}$ M $T_3$ (table 2). Similar results were obtained in mouse L fibroblasts (table 2). The renin sequences of ptR2CAT were also placed upstream of the SV40 early promoter (either directly upstream or separated by an UMS element). No significant effect of renin sequences on CAT activity could be seen after transfection in T47D cells. In fact, insertion of renin sequences upstream of the SV40 early

Table 2

Expression of 'hybrid promoter'-CAT constructs

| Plasmid | Cell line | | | |
|---|---|---|---|---|
| | T47D | GC | L | BHK |
| pttkCAT | 0.40 | 1.35 | 0.13 | ND |
| ptR2tkCAT | 0.34 | 1.05 | 0.10 | ND |
| ptSVECAT | 0.6 | ND | 1.9 | 10 |
| ptR2tSVECAT | 1.3 | ND | 2.8 | 26 |
| ptR2SVECAT | 3 | ND | 2.1 | 13 |

CAT activity was determined on 0.2–0.3 mg of cytosolic protein 65 h after transfection. It is expressed as % of acetylated chloramphenicol during a 2-h incubation. Numbers are the mean of two to four experiments. ND, not determined

promoter resulted in a slight increase in CAT activity (1–3-fold) and was also obtained in BHK or L fibroblasts. Thus, in two different series of experiments, we could not see any striking effect of *Ren*-2 5'-flanking sequences on the activity of a heterologous promoter nor could we observe activity of the thyroid hormone response element in cells that possess the appropriate receptor.

## 4. DISCUSSION

Tissue-specific expression of a number of genes is conferred by enhancer elements that have been localized in their vicinity. The immunoglobulin genes [24–27], the gene for insulin [28], chymotrypsin [28], the prolactin and growth hormone genes [29] and the δ1-crystallin gene [30] represent only a few examples. In most of these cases, the enhancer was able to confer tissue specificity to a heterologous promoter. A direct role for promoter elements in tissue-specific expression has been demonstrated for immunoglobulin genes [26,31,32] and possibly insulin [33] and α-fetoprotein [34,35]. In the present work, we show that even though expression of the mouse renin genes is limited to a few tissues, i.e. mainly the juxtaglomerular cells of the kidney and the tubular cells of the submaxillary gland as well as a few other extra-renal sites, the promoters of *Ren*-1 and *Ren*-2, defined as the elements necessary for accurate initiation of transcription, do not function in a tissue-specific manner. Indeed, when coupled to the SV40 enhancer, the promoters of the two genes can direct transcription of the indicator CAT

gene in BHK, L, CHO, JEG-3 and T47D cells, none of which normally express the renin gene. A similar phenomenon has been described for other mammalian genes, including the lysozyme [36], conalbumin [18], $\delta$1-crystallin [29]. The only cells where the renin sequences are inactive even when coupled to a functional enhancer are lymphoid cells [8]. In these cells, a renin promoter becomes active only after insertion of the conserved decanucleotide element which confers B-cell specificity to the promoters of the immunoglobulin heavy and light chain genes. This effect of the decanucleotide seems to be limited to cells of the B lineage because constructions containing the decanucleotide are no more active than those with the original renin promoter in T3T cells [8], CHO and T47D cells (our unpublished observations).

Promoter activity is obtained with fragments that span 2.5 kb of *Ren-2* upstream sequence as well as 500 or 180 bp of *Ren-1*. Overall, the activities obtained with all fragments are roughly equal, that is reducing the size of the renin fragment does not lead to any significant increase in activity. Therefore it appears unlikely that an element with repressor-like properties is present within the upstream region of the renin genes and that tissue-specific control of renin gene expression is achieved through repression. The absence of CAT activity, when ptR2CAT is transfected into cells that do not normally express the renin gene, is then probably due to the absence from these cells of one or several *trans*-acting positive factor(s) [8,37].

The present experiments lead to the suggestion of a model for the transcriptional regulation of the renin gene. First, no constraints are put on the promoter, that is, the main factor is not whether transcripts can be initiated correctly in a given cell type but rather whether they can be initiated in sufficient numbers. This is consistent with expression of the renin genes in various extra-renal sites without necessity for very complex regulatory mechanisms (Ekker et al., Proc. Natl. Acad. Sci. USA, in press). Secondly, although not demonstrated here, the presence of an enhancer-like element that functions in a tissue-specific manner is probable. The renin genes are expressed in more than one cell type and thus the possibility of several distinct elements of this kind is not excluded. Assuming that one of these elements is present in the 5'-flanking region of the renin genes, its lack of effect on hetero-

logous promoters (tk, SV40; table 2) further supports a possible tissue-specific activity. Finally, the enhancement of transcription resulting from the binding of an inducer-receptor complex to its target sequence could be dependent upon the proper function of the tissue-specific enhancer. Such mechanism would, for example, prevent unwanted increase of the transcription of the renin genes in other cells that are responsive to the inducer but do not normally produce renin.

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