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Identification of a negative regulatory element involved in tissue-specific expression of mouse renin genes

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ABSTRACT The 5′ flanking region of the mouse renin genes (*Ren-1^d^* and *Ren-2^d^*) contains two motifs that are homologous to known negative regulatory elements (NREs). *Ren-2^d^* has a 150-base-pair (bp) insertion 5′ to the upstream putative NRE (NRE-1), which is lacking in *Ren-1^d^*. We tested the functionality of these sequences by using site-directed mutagenesis to delete individually each putative NRE from *Ren-1^d^* and to delete the 150-bp insertion from *Ren-2^d^*. We examined the effect of these mutations on the expression of the reporter gene chloramphenicol acetyltransferase, which was expressed from a truncated thymidine kinase promoter fused to the renin regulatory region. This plasmid was transfected into human choriocarcinoma JEG-3 cells. Only the upstream NRE (positions −619 to −597) was found to be functional in *Ren-1^d^*. The deletion of a 150-bp insertion from *Ren-2^d^* resulted in the suppression of chloramphenicol acetyltransferase activity to the level of *Ren-1^d^* expression. These data suggest that the upstream NRE that is functional in *Ren-1^d^*, but not in *Ren-2^d^*, may be partly responsible for differential expression of the renin genes in various tissues. The molecular mechanism of the NRE was examined by studying its interaction with nuclear proteins in submandibular gland and JEG-3 cells by gel-mobility-shift assays. Specific nuclear protein binding was observed only to the upstream NRE and the molecular mass of this protein was ≈72 kDa as determined by Southwestern blot analysis. Thus our results suggest that both *Ren-1^d^* and *Ren-2^d^* conserve a cis-acting NRE in the 5′ flanking region. In *Ren-1^d^*, this NRE could bind a specific nuclear protein resulting in the inhibition of *Ren-1^d^* expression in these tissues. On the other hand, the NRE in *Ren-2^d^* is nonfunctional due to interference by an adjacent 150-bp insertion.

Renin is produced within the juxtaglomerular cells of the kidney, where it is stored in granules and released into the circulation. Circulatory renin has a well-established role in blood pressure and volume homeostasis (1, 2). Renin is also produced in a variety of other tissues, including submandibular gland (SMG), adrenal gland, heart, testis, and ovary. Tissue renin regulates the production of local angiotensin, which exerts paracrine/autocrine influences on local tissue functions. However, little is known about the control of extrarenal expression of the renin gene. To investigate the tissue-specific gene expression, we have chosen the DBA/2J mouse, which carries two renin loci, *Ren-1^d^* and *Ren-2^d^*. These genes are homologous in structure including their 5′ flanking regions but differ markedly in their pattern of tissue expression. They are expressed equally in the kidney. However, in the SMG, *Ren-1^d^* is silent and *Ren-2^d^* is expressed at the same level, on a per expressing cell basis, as in the kidney (3). Previously, we compared the functionality of 5′ flanking sequences of the two genes in JEG-3 cells (human choriocarcinoma), which are derived from the chorion, a renin-producing tissue (4). We chose to use the truncated thymidine kinase promoter of pUTKAT to enhance expression in JEG-3 and to study the modulation of this expression by the upstream mouse renin sequences. We found that a 340-base-pair (bp) *Xba* I fragment from *Ren-1^d^* could suppress basal expression from a heterologous promoter by ≈60%. The corresponding *Xba* I fragment of *Ren-2^d^*, which differs from *Ren-1^d^* by the presence of a 150-bp insertion, did not suppress expression. Both fragments contained two sequences that were homologous to two regions of the chicken lysozyme silencer (5) and that we termed putative negative regulatory elements (NREs). These two putative NREs were not homologous to each other. The upstream NRE (NRE-1) is located at a position immediately 3′ to the 150-bp insert in *Ren-2^d^*, and the other NRE (NRE-2) is located 60 bp downstream of this site.

In the present study, we tested the hypothesis that the upstream putative NRE acts as a functional NRE in *Ren-1^d^* but is nonfunctional in *Ren-2^d^* due to the presence of the 150-bp insertion.

MATERIALS AND METHODS

Cell Lines and Cultures. JEG-3 cells (American Type Culture Collection HTB-36) used for transfection studies are of human choriocarcinoma origin. They were grown as a monolayer in Eagle's minimal essential medium with 10% (vol/vol) fetal calf serum, in an atmosphere of 5% CO_2/95% air.

Plasmid Constructions. pUTKAT contains a truncated herpes simplex virus thymidine kinase promoter that directs the transcription of a chloramphenicol acetyltransferase (CAT) gene (6). To test the effect of renin 5′ sequences (Fig. 1*A*) on transcription in this system, a 340-bp *Xba* I fragment from the *Ren-1^d^* gene (positions −707 to −367) and the corresponding 484-bp *Ren-2^d^ Xba* I fragment were cloned upstream of this promoter (Fig. 1*B*). Deletional mutations (Fig. 1*B*) of these renin segments were constructed by the PCR using the overlap-extension method (7), which uses PCR primers to "loop out" the desired sequences. This technique utilizes complementary oligonucleotide primers comprising sequences on either side of, but omitting, the sequences to be deleted. These primers were used in conjunction with flanking primers to generate two DNA fragments having overlapping ends. These fragments are then combined in a subsequent "fusion" PCR to regenerate the original fragment but incorporating the specific deletions. The 150-bp segment CACCTAAG. . .AAGTAAAA (which occurs as an insert in *Ren-2^d^* at a position corresponding to nucleotide −619 of *Ren-1^d^*) was deleted from *Ren-2^d^* to create Ren2^{d}Δ150, and

Abbreviations: SMG, submandibular gland; NRE, negative regulatory element; CAT, chloramphenicol acetyltransferase; CRE, cAMP-responsive element; hCG, human chorionic gonadotropin.
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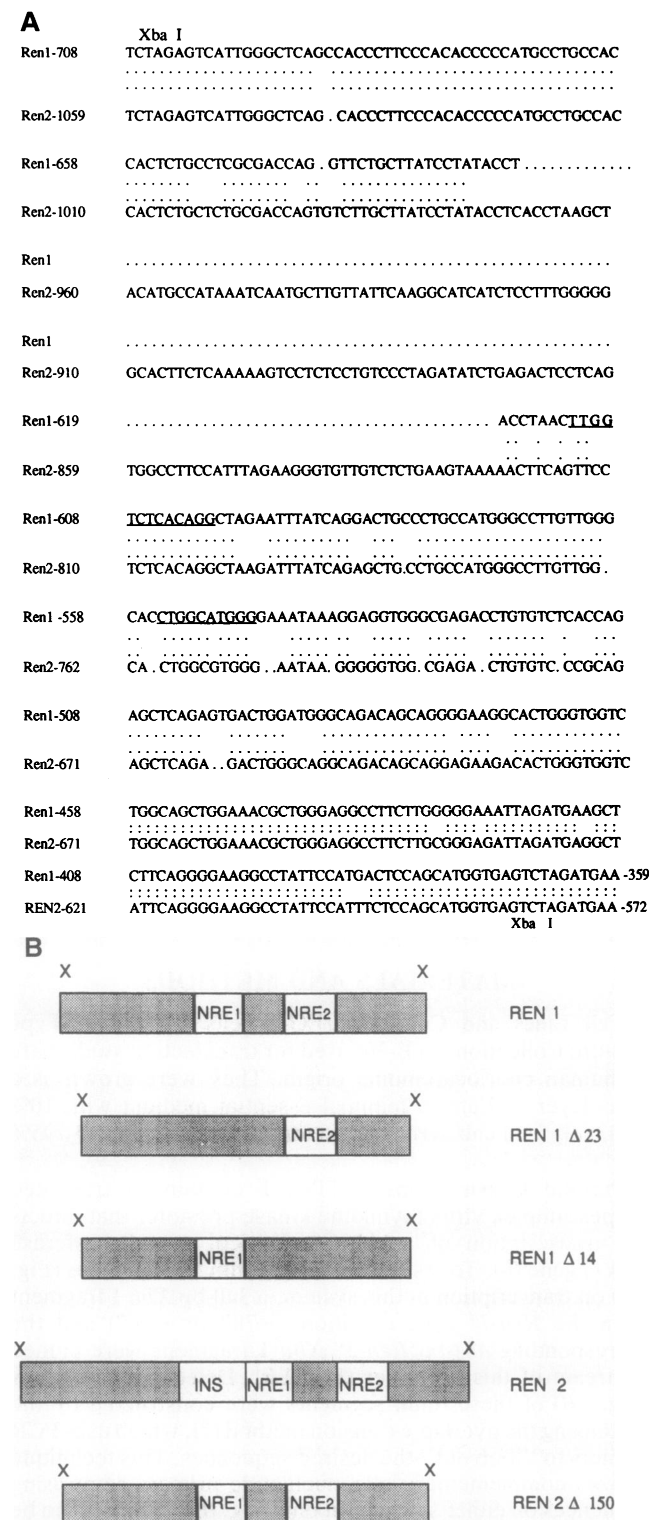

FIG. 1. (*A*) Alignment of 5′ flanking regions of mouse *Ren-1^d^* and *Ren-2^d^* genes, showing the *Xba* I fragments of both genes. The putative NREs are underlined in *Ren-1^d^*, and the 150-bp insertion in *Ren-2^d^* is shown. (*B*) Wild-type and mutagenized renin 5′ flanking sequences used for reporter gene constructs. A 23-bp segment including the putative upstream NRE (NRE-1) was deleted by directed mutagenesis from the 340-bp *Xba* I fragment of *Ren-1^d^* to obtain Ren1^{d}Δ23. A 14-bp segment including the putative downstream NRE (NRE-2) was deleted from the same *Ren-1^d^* fragment to obtain Ren1^{d}Δ14. The 150-bp insertion in *Ren-2^d^* was deleted from the 480-bp *Xba* I fragment of *Ren-2^d^* to obtain Ren2^{d}Δ150. Wild-type and mutagenized *Xba* I fragments were fused in the positive orientation to the *Xba* I site immediately upstream of the truncated thymidine kinase promoter in pUTKAT. INS, 150-bp insertion; X, *Xba* I.

the 23-bp segment TACCTAACTTGGTCTCACAGGCT (positions −619 to −597) of *Ren-1^d* was deleted to create Ren1^{d}Δ23. The 14-bp segment of *Ren-1^d* ACCTGGCATGGGGA (positions −557 to −544) was deleted to create Ren1^{d}Δ14. The mutagenized fragments were subcloned into M13 and sequenced to assure generation of the desired mutant.

Transfection and CAT Assay. JEG-3 cells at 80% confluence were transfected with plasmid DNA (10 μg) by the calcium phosphate method (8). Forty-eight hours later, cells were collected with trypsin/EDTA and assayed for CAT activity (9). CAT assays were quantitated by scintillation counting after separation of chloramphenicol and acetylated chloramphenicol by thin layer chromatography. Results were expressed relative to the level obtained with pUTKAT [6.4% acetylation per 100 μg of protein (10)].

Preparation of DNA Probe. For DNA–protein interaction studies, a 32-bp probe, spanning the upstream putative NRE, was made by synthesizing and annealing the 32-mer oligonucleotide and its complement:

5′-gatcCTAACTTGGTCTCACAGGCTAGAATTTA-3′

3′-GATTGAACCAGAGTGTCCGATCTTAAATagct-5′

(where lower case letters are noncomplementary sequences added to assist in labeling).

Annealing was performed by cooling from 80°C to room temperature over 1 h. After annealing, the 32-mer was ^{32}P-labeled by end-filling with the Klenow fragment of DNA polymerase I. Similarly, a 21-bp probe, spanning the downstream putative NRE, was made by annealing the oligonucleotide 5′-gatcGCACCTGGCATGGGGAA-3′ with its complement.

Unlabeled versions of each of these probes were also used as unlabeled competitors for DNA binding in the incubation mixtures. Two other double-stranded oligonucleotides were used as competitors in the gel-retardation assays. An oligonucleotide was made to the putative cAMP-responsive element (CRE) in the *Ren-1^d* gene, CTATAACCTACCTAACTTGGTCTCACAGG, which overlaps with the NRE sequence. An oligonucleotide was also made to the "classical" CRE of the human chorionic gonadotropin (hCG) α subunit gene, CAAATTGACGTCATGGTAAT.

Preparation and Analysis of Nuclear Extract. Nuclear extract from JEG-3 cells was prepared using a protocol adapted from Dignam *et al.* (11) and SMG nuclear extract was prepared as described by Lee *et al.* (12). The gel-mobility-shift assays (13) and Southwestern blot analysis (14) were performed as described.

RESULTS

Our laboratory has demonstrated (4) the presence of a silencer within the 340-bp *Xba* I fragment of the *Ren-1^d* 5′ regulatory region, which was functional in JEG-3 and AtT-20 cells. Based on sequence comparisons with the silencer regions of the chicken lysozyme gene (5), we identified two possible silencer regions, a 13-bp sequence beginning at position −612 (relative to the cap site) and a 9-bp sequence, at position −555, referred to as "upstream putative NRE" and "downstream putative NRE," respectively. We tested the functionality of these sequences by using site-directed mutagenesis to delete individually each sequence from *Ren-1^d* and measured the effect of these deletions on gene expression. As in our previous findings, the expression of *Ren-2^d*–pUTKAT did not differ from that of pUTKAT, whereas *Ren-1^d*–pUTKAT expression was 60% lower than that of pUTKAT. The expression of Ren1^{d}Δ23–pUTKAT, in which the upstream putative NRE was deleted, was 75%

higher than that of *Ren-1^d*–pUTKAT (Fig. 2 *A* and *B*). The level attained with Ren1$^d\Delta$23–pUTKAT was not different statistically from that of pUTKAT, indicating that this construct did not contain a functional NRE. Expression of Ren1$^d\Delta$14–pUTKAT, in which the downstream putative NRE was deleted, was unaltered with respect to *Ren-1^d*–pUTKAT and was 50% less than pUTKAT, indicating that this construct retained a functional NRE (data not shown). We conclude that only the upstream NRE is functional in JEG-3 cells.

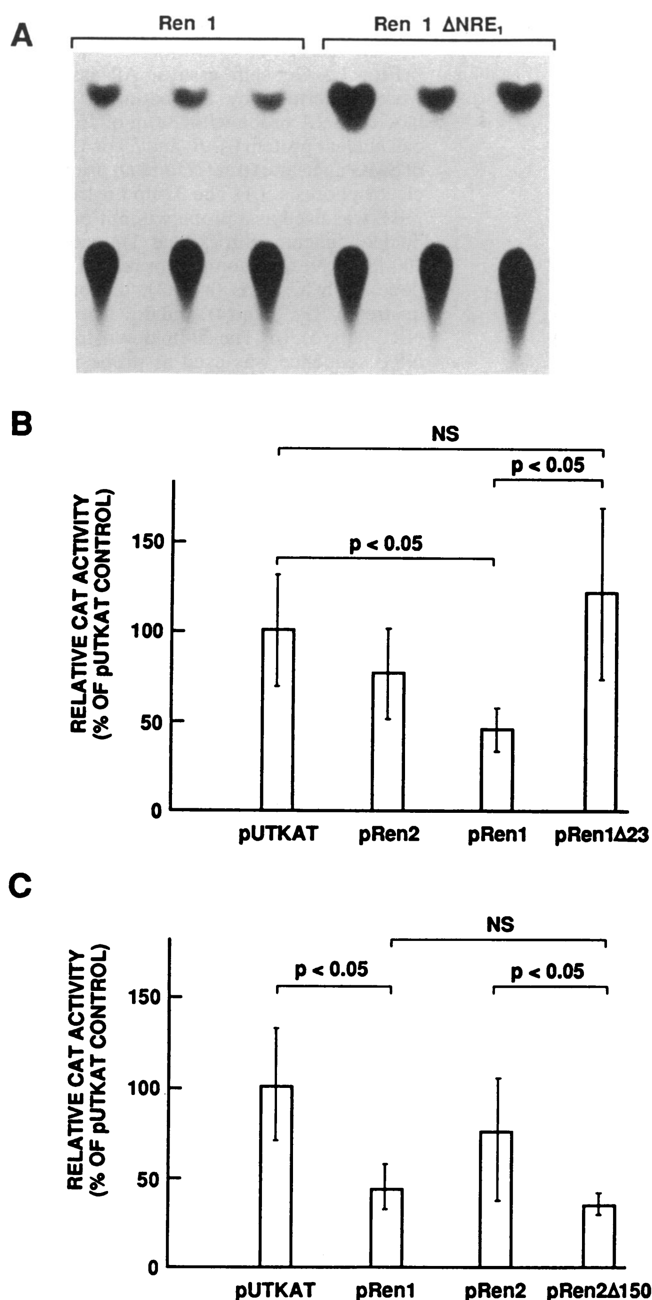

FIG. 2. CAT activity in JEG-3 cells transfected transiently with pUTKAT–renin gene fusion constructs. Each transfection used 10 μg of purified supercoiled plasmid and was carried out on 2×10^6 cells at 80% confluence, using the calcium phosphate method (10). Cells were harvested for CAT assay 48 h after transfection. Percentage acetylation was calculated after measuring the radioactivity of acetylated and nonacetylated chloramphenicol molecules, which had been separated on TLC plates. (*A*) Representative autoradiography showing enhanced acetylation in cells transfected with Ren1$^d\Delta$23, in which the upstream putative NRE was deleted (Ren1ΔNRE$_1$), compared to wild-type *Ren-1^d*. Results of this experiment are summarized in *B*. (*B*) Acetylation resulting from Ren1$^d\Delta$23 is unaltered from that of pUTKAT, indicating that Ren1$^d\Delta$23 does not contain an NRE. (*C*) Deletion of 150-bp insert of *Ren-2^d* results in a reduction of acetylation from the level of pUTKAT to that of *Ren-1^d*. In both experiments, results were compiled after seven transfections of each plasmid. Data are mean ± SD. Tests of statistical significance were performed by Student's *t* test. NS, not significant.

The 13-bp putative NRE sequence of *Ren-1^d* is also present in *Ren-2^d* but is located adjacent to a 150-bp insertion that is not present in *Ren-1^d*. We constructed a mutant, Ren2$^d\Delta$150, in which the 150-bp insert was deleted from *Ren-2^d*. Transfection of Ren2$^d\Delta$150–pUTKAT into JEG-3 cells resulted in a reduction of CAT expression by 60% compared to *Ren-2^d*–pUTKAT (Fig. 2*C*). This result suggests that the 150-bp insertion in *Ren-2^d* interferes with the function of the NRE.

Nuclear protein extracts of JEG-3 cells were used to investigate DNA–protein interactions at the putative NRE sequence. As shown in Fig. 3*A*, a specific nuclear protein–DNA complex was observed that was competed by the upstream NRE oligonucleotide but not by mouse renin CRE (which overlaps NRE partially), the downstream NRE, or the hCG CRE. The 20-bp downstream putative NRE was also radiolabeled and used as a probe. Although a weak retarded band was observed, it was presumably nonspecific since all the competitive oligonucleotides blocked the formation of this band (Fig. 3*B*). To test for the presence of this NRE binding protein in SMG, the tissue in which *Ren-1^d* is suppressed and *Ren-2^d* is expressed, we examined the nuclear protein–DNA complex formation by using a *Xba* I fragment of *Ren-1^d* and *-2^d* as a ^{32}P-labeled DNA probe (Fig. 3 *C* and *D*). As shown in Fig. 3*C*, SMG nuclear extract showed nuclear protein–DNA complex formation that was competed by the addition of unlabeled upstream NRE but not by mouse renin CRE and hCG CRE oligonucleotides. Although we observed SMG nuclear protein–*Ren-2^d* complex formation, this binding was not competed by upstream NRE oligonucleotide, suggesting that the NRE specific nuclear binding protein cannot bind to the NRE in *Ren-2^d*. To confirm that the 150-bp insertion of *Ren-2^d* interfered with the NRE binding, we performed a further competition analysis using JEG-3 cell nuclear extract and a ^{32}P-labeled NRE oligonucleotide. As shown in Fig. 3*E*, the *Xba* I fragment of *Ren-1^d* and Ren-2$^d\Delta$150 interfered with the NRE binding protein–NRE binding. However, the *Xba* I fragment of *Ren-2^d* did not inhibit the binding.

Nuclear proteins extracted from JEG-3 cells were separated by SDS/polyacrylamide electrophoresis, transferred to nitrocellulose, and probed with the upstream ^{32}P-labeled NRE oligonucleotide. A strong band was observed at 72 kDa, and a weaker one at 33 kDa (Fig. 4*A*). We also performed Southwestern blot analysis on nuclear extracts prepared from mouse SMG. The 72-kDa band was clearly present, but the 33-kDa band was absent (Fig. 4*B*).

Recent studies have described a number of gene regions that negatively regulate expression. In most cases, the specific sequences responsible for the negative regulation have not been defined but have been localized to a restriction or exonuclease fragment. We compared the mouse renin NRE sequence to published sequences of silencer fragments (Fig. 5). Homologous motifs were found within a negative regulatory fragment of the human renin gene (15), the rat collagen II gene (16), two separate silencers described for the human T-cell receptor gene (17), and the chicken lysozyme gene (5). Additionally, a homologous motif occurs in the opposite orientation within a silencer region of the c-*myc* gene (18). Based on a comparison of these sequences, we propose a 13-bp consensus sequence of the NRE (Fig. 5). The 5′ flanking region of the rat renin gene contains a homologous sequence, TTGGCCTCACGGG, that differs from the mouse

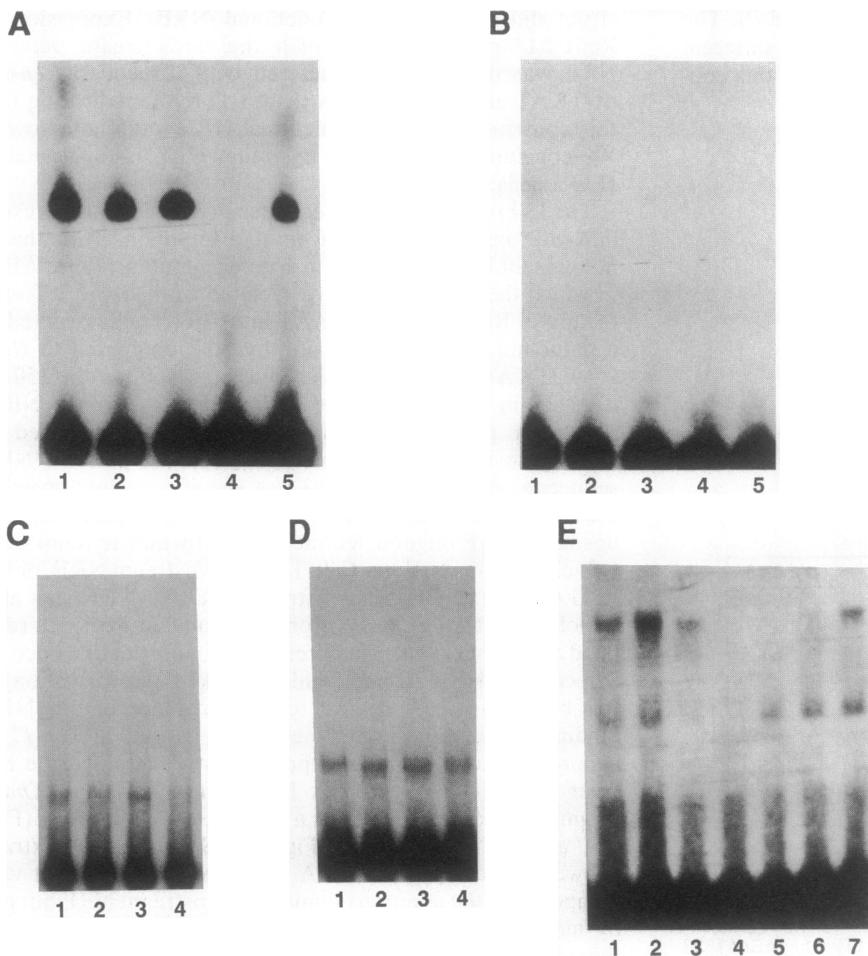

FIG. 3. Gel-shift assays. All assays were performed by incubating a ^{32}P-labeled DNA probe with 20 μg of JEG-3 cell nuclear protein (*A*, *B*, and *E*) or 15 μg of SMG nuclear extract (*C* and *D*) prior to electrophoresis. (*A*) The 32-bp upstream NRE was used as a probe without oligonucleotide competitor (lane 1), with a 100-fold excess of competitor renin CRE (lane 2), hCG CRE (lane 3), unlabeled upstream NRE (lane 4), and downstream NRE (lane 5). (*B*) The 20-bp downstream NRE sequence was used as probe with and without oligonucleotide competitors. No specific bands were seen. Lanes are as in *A*. (*C*) The *Xba* I fragment of *Ren-1^d* was used as a probe without oligonucleotide competitor (lane 1), and with a 100-fold excess of competitor renin CRE (lane 2), hCG CRE (lane 3), and upstream NRE (lane 4). (*D*) The *Xba* I fragment of *Ren-2^d* was used as a probe without oligonucleotide competitor (lane 1), and with a 100-fold excess of competitor renin CRE (lane 2), hCG CRE (lane 3), and upstream NRE (lane 4). (*E*) The NRE oligonucleotide was used as a DNA probe without competitor (lane 1) and with 100-fold excess of unlabeled renin CRE (lane 2), hCG CRE (lane 3), and upstream NRE (lane 4), and *Xba* I fragments of *Ren-1^d* (lane 5), *Ren-2^d* (lane 7), and Ren2Δ150 (lane 6).

renin NRE by only two nucleotides (19), but functional studies for this gene have not been reported.

DISCUSSION

Our previous work demonstrated the existence of a NRE in the *Ren-1^d* gene that is nonfunctional in the *Ren-2^d* gene (4). We now show that the suppression exerted by the *Ren-1^d Xba* I fragment can be abolished by the deletion of a specific 23-bp sequence and that this DNA sequence binds specifically to a nuclear protein present in JEG-3 cells. This nuclear protein was shown by Southwestern blot analysis to be ≈72 kDa and to be present in the SMG. Differential expression of *Ren-1^d* and *Ren-2^d in vivo* is most marked in SMG and thus it would have been preferable to perform functional studies in SMG cells. This was not undertaken due to the lack of a suitable clonal cell line.

The degree of *in vitro* suppression (i.e., 60%) in JEG-3 cells caused by the renin NRE is low compared to the *in vivo* suppression of *Ren-1^d* expression (compared to *Ren-2^d*) in SMG, which is at least 99%. This may be due to differences

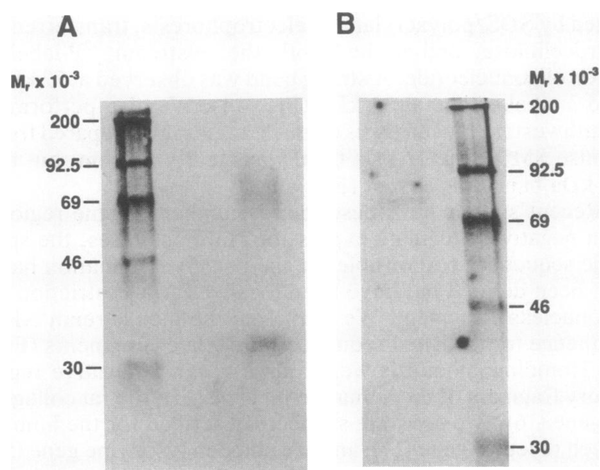

FIG. 4. Southwestern blot analysis. (*A*) Nuclear proteins from JEG-3 cells were probed with the 32-bp upstream NRE sequence in presence of 100-fold excess of salmon sperm DNA. Bands were seen at 33 kDa and 72 kDa. (*B*) Nuclear proteins from mouse SMG were treated as in *A*, yielding an 72-kDa band and a faint larger band.

Mouse Ren1^d	ctaac	TTGGTCTCACAGG	ctagaa
Human Renin	ctagc	TCTGTCCCGCAGT	gagatt
TCR Sil I	aggga	TATTTCCCAGAGG	tgagtg
TCR Sil II	agagg	TCATTCCAAGAGG	ttgaat
c-Myc	atgat	TTATACT CACAGG	acaagg
Collagen II*	tgtat	TCCTACACAGAGG	gggacc
Chick Lysozyme	gaact	CAGGTCTCTCAGG	ctactg
Consensus		TNNTTCCCACAGG G T G	
Downstream NRE		CTGGCATGGG	

(*Non-coding strand)

FIG. 5. Alignment of *Ren-1^d* NRE sequence with sequences from silencers and negative regulatory regions of other genes. Based on this alignment, a provisional consensus sequence of the NRE was constructed. Only two positions are truly invariant, but five other positions show a preference for a particular nucleotide. Three positions have a preference for either of two nucleotides, and two positions (the second and third positions) do not appear to have any nucleotide preference. TCR, T-cell receptor; Sil, silencer.

in trans-acting factors between the two tissues. However, a 72-kDa nuclear protein capable of binding the NRE oligonucleotide was found in JEG-3 cells and in SMG. The lower degree of suppression may also be due to the limitations inherent in using CAT assays as an index of transcription (20). However, it is possible that other sites in the *Ren-1^d* gene are also involved in silencing and cooperate with the NRE described herein to cause more profound suppression. The few cases where negative regulatory regions (often called silencers) of other genes have been characterized in depth reveal that effective silencing requires protein binding at multiple sites. These sites may contain closely related sequences in some cases and quite different sequences in other cases. Negative regulation of the human insulin gene involves a number of sites in close proximity that may facilitate cooperative binding of trans-acting factors (21). Various NREs within the chicken lysozyme gene have recently been described and studied (5, 22). When fused upstream of heterologous regulatory sequences, individual NREs were found to cause a small suppression whereas concatamerized NREs caused a much greater suppression. If the renin gene contains other negative elements, however, such sites are probably not within the 340-bp *Xba* I fragment as deletion of a specific 23-bp region alone abolished the ability of this fragment to suppress expression from pUTKAT. A candidate sequence, the downstream putative NRE, was shown by functional and binding studies not to be important. It remains possible, however, that other sites exist within the *Xba* I fragment that are functional in SMG but not in JEG-3 cells.

The region of the 23-bp deletion was chosen for its homology to a region within a chicken lysozyme silencer element (5). Subsequently, silencer regions have been found in various genes, and the NRE motif occurs in several of these genes including collagen II (16), the T-cell receptor (17), c-*myc* (18), and human renin (15). These diverse genes show marked tissue-specific expression. If the same NRE is conferring tissue specificity in these genes, it is difficult to explain their expression in such different tissues. One possibility is that silencing requires the interaction of a number of trans-acting factors, as discussed above. The presence of an NRE-binding protein within a given tissue may be necessary but not sufficient to silence genes containing the NRE sequence. Other negative trans-acting factors may also be required, and these may differ among the various genes containing the NRE sequence. In this model, a relatively small number of trans-acting factors would allow different tissue specificities of expression for a number of different genes.

A further question relates to how this NRE interacts with the promoter. The best-known example is RAP-1 in yeast, a transcription factor that is involved in suppressing mating-type promoters at two loci, *HMR* and *HML*. At least one other protein (and DNA-binding site) is required for suppression. Interestingly, it has been shown that RAP-1 can cause DNA-loop formation at the *HML* locus *in vitro*, either by silencer–silencer or silencer–promoter interactions (23). Thus, RAP-1 binding may induce chromatin loops that maintain the promoter in an inactive state. RAP-1 is also capable of binding to the nuclear matrix. Immunoglobin heavy chain locus positive and negative elements may also be associated with chromosomal loop anchorage elements (24). Negative regulation can also occur by competition with a positive factor for a single site (e.g., *jun-B* vs. c-*jun*) (25).

In conclusion, we have shown that renin genes contain a sequence capable of suppressing expression in JEG-3 cells and that a specific interaction occurs between this DNA sequence and a protein present in JEG-3 cells. The presence of a 150-bp insert adjacent to this site in *Ren-2^d* can inactivate the NRE, allowing a greater level of expression. These sequences may be involved in the differential expression of the renin genes in SMG. The observation of an NRE that is functional in *Ren-1^d* but not in *Ren-2^d* supports the hypothesis of Field and Gross (26) that the high expression of *Ren-2^d* (compared to *Ren-1^d*) in SMG is due to nonresponsiveness of *Ren-2^d* to negative control. In this case, escape from negative control has occurred by means of a 150-bp insertion that probably interrupts binding of a nuclear factor to the NRE sequence as shown in Fig. 5. It is also a remote possibility that the 150-bp insertion of *Ren-2^d* contains an enhancer element. However, the insertion does not show homology to known sequences (19). Confirmation of the role of the NRE and *Ren-2^d* insertion sequence in SMG renin expression will require functional studies in SMG tissue, either by development of an SMG cell line from the DBA mouse and subsequent transfection studies or by analysis of expression of renin gene mutants in transgenic mice.

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