MOLECULAR CLONING OF DNA COMPLEMENTARY TO MOUSE SUBMANDIBULAR GLAND RENIN mRNA

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ABSTRACT

A recombinant plasmid containing a DNA segment complementary to the mRNA for mouse submandibular gland renin has been constructed and cloned. Renin mRNA was enriched by poly(U)-Sepharose chromatography and sucrose density gradient centrifugation. Double-stranded cDNA, prepared from this enriched mRNA with reverse transcriptase and DNA polymerase I, was inserted into the *Pst* I site of pBR322 by the dC-dG tailing technique. Recombinant plasmids containing this cDNA were amplified in *Escherichia coli* χ 1776. One clone containing a 700-base pair insert complementary to renin mRNA was isolated by positive selection. Southern blot hybridization of genomic DNA following restriction enzyme digestion and gel electrophoresis demonstrated that the mouse renin cDNA cross-hybridizes with renin genes of various mammals including humans.

Recombinant DNA technology allows cloning of DNA fragments for application as hybridization probes in many areas of research. In the preceding paper (5), as a first step in preparing cDNA probes useful in studying the androgen-dependent biosynthesis of mouse submandibular gland renin, we isolated poly(A)-containing RNA from this gland and analyzed its translation products. In this paper, we report the construction and cloning in *E. coli* of a cDNA fragment for renin.

MATERIALS AND METHODS

Parts of the reagents and animals are described in the preceding paper (5). Materials were obtained as follows: terminal deoxynucleotidyl transferase (terminal transferase) from Bethesda Research Laboratories; restriction endonucleases from Takara Shuzo; *SI* nuclease from Sankyo; *E. coli* DNA polymerase I (the large fragment) from Boehringer Mannheim; oligo(dT)₁₀ primer

from Miles Laboratories; radiochemicals and nick translation kits from Amersham. Avian myeloblastosis virus reverse transcriptase was a kind gift from Dr J. W. Beard of Life Sciences, Inc. Plasmid pBR322 was isolated as described by Matsubara *et al.* (6). Genomic DNAs from rat liver, mouse liver, calf thymus, and human placenta were kind gifts from Dr N. Okada (University of Tsukuba).

RNA Preparation, Cell-Free Translation, and Immunoprecipitation

RNA preparations from the submandibular glands of adult male mice were carried out as described (5). The cell-free translation in a rabbit reticulocyte lysate system and immunoprecipitation of the radioactive protein were also done as described (5). Double-stranded cDNA was synthesized using the conditions described by Crabtree and Kant (2). RNA fractions containing renin mRNA were used for reverse transcription by using avian myeloblastosis virus reverse transcriptase. The cDNA, after heat treatment, was rendered double-stranded by using *E. coli* DNA polymerase I. The cDNA

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was treated with S1 nuclease and, after phenol extraction and gel chromatography on Sephadex G-100, the nucleic acids eluted in the void volume were ethanol precipitated. Homopolymer dC tails were added by using terminal transferase at 37°C for 10 min (8). On the average, 25 dCMP residues were added to each 3'-OH end. The circular pBR322 DNA was digested by endonuclease *Pst* I and homopolymer dG tails were added to the linear DNA. An average of 10 dGMP residues were added to each 3'-OH end. The double-stranded cDNA that had been tailed with poly(dC) (100 ng) was hybridized to 800 ng of pBR322 that had been tailed with poly(dG) (12) and was then used to transform *E. coli* cells.

Transformation of *E. coli*

The procedure of Villa-Komaroff *et al.* (13) was used to transform strain χ 1776 with the hybridized DNA mixture described above. Colonies that were resistant to tetracycline at 10 μ g/ml and sensitive to ampicillin at 50 μ g/ml were selected and screened for the presence of renin sequences by using the positive hybrid selection assay described below. These experiments were carried out in a P2 biocontainment facility at the University of Tsukuba in compliance with the guidelines of the Ministry of Education, Science and Culture, Japan on recombinant DNA research.

Positive Hybrid Selection Assay

Positive hybrid selection assay was performed

according to the method of Parnes *et al.* (10), except that plasmid DNA was extracted using the method of Birnboim and Doly (1). Eluted RNA was translated in the cell-free translation system described above.

Restriction Mapping

The plasmid containing renin cDNA was cleaved with several restriction enzymes using the buffers described by Davis *et al.* (3). The cleaved products were separated by 5% polyacrylamide gel electrophoresis (7). After staining, the gel was photographed on a transilluminator.

Southern Blot Hybridization

Genomic DNAs were digested with *Eco* RI and electrophoresed on 1% agarose gels as described above. DNAs were transferred overnight onto nitrocellulose membrane filters according to the procedure described by Southern (11). Hybridization with appropriate probes was done as described by Dawid (4). Nick-translations of probes were done as recommended by the supplier.

RESULTS

Preparation of cDNA

Double-stranded cDNA was synthesized from the partially purified renin mRNA by a protocol summarized in Table 1. The first and second strands of cDNA were synthesized in the same

Table 1 *Synthesis and Cloning of cDNA*

A. Double-stranded cDNA synthesis			
Step			Yield
cDNA synthesis (from 10 μ g of mRNA)			284 ng
ds cDNA synthesis			686 ng
S1 nuclease digestion of ds cDNA			481 ng
B. Cloning of ds cDNA			
Clone phenotype	DNA used for transformation		Number of transformant
Tc ^r	pBR322 native DNA	1 ng	1,230
	pBR322-dG-tailed	800 ng	653
	+ ds cDNA-dC-tailed	100 ng	
Tc ^r Ap ^s	pBR322-dG-tailed		106
	+ ds cDNA-dC-tailed		

Tc^r, tetracycline resistant; Ap^s, ampicillin sensitive

tube without ethanol precipitation between the steps to improve the yield. Using DNA polymerase I, we obtained 60 to 70% resistance to S1 nuclease. About 480 ng of double-stranded cDNA remained after S1 treatment.

dC-dG Tailing and Transformation

Double-stranded cDNA molecules were inserted into the *Pst* I site of pBR322 by the oligo(dC)-oligo(dG) annealing procedure. The plasmid pBR322 contains ampicillin and tetracycline resistant loci with the single restriction site for *Pst* I in the former. Thus, the construction of hybrid cDNA-pBR322 molecules in this way

allows 1) detection of recombinants by screening for an ampicillin-sensitive, tetracycline-resistant phenotype ($\text{amp}^s \text{tet}^r$) and 2) excision of the inserted DNA with *Pst* I since the *Pst* I site is regenerated at both vector-insert junctions. The annealing mixture was used to transform the χ 1776 strain of *E. coli* to tetracycline resistance; approximately 653 tet^r colonies were obtained from 100 ng of tailed cDNA. Tetracycline-resistant transformants were then screened for ampicillin sensitivity; 106 $\text{amp}^s \text{tet}^r$ colonies were selected. This number of transformant clones was considered adequate to isolate several renin-cDNA clones since renin mRNA was enriched to a concentration of 5–10% of the total mRNA

Fig. 1 Hybrid selection and cell-free translation of mRNA encoding precursor of mouse submandibular gland renin. Plasmid DNAs containing renin cDNA were immobilized on nitrocellulose filters (Millipore 0.45- μm pores) and used to select, by hybridization renin specific mRNA from poly(A)⁺RNA fraction, purified by sucrose density gradient centrifugation (5). mRNA was then eluted from the DNA-RNA hybrid. mRNAs so selected were translated in a rabbit reticulocyte lysate system containing [³⁵S]methionine. The labeled products were analyzed by immunoprecipitation and 10% polyacrylamide gel containing sodium dodecyl sulfate. An autoradiograph was prepared from the dried gel. The DNAs immobilized on the nitrocellulose were: Lane 1, pBR322 only; Lanes 2 and 3, cDNA-pBR322 recombinants lacking renin sequence; Lane 4, renin cDNA-pBR322 recombinant. Lane 5: as control of the mobility of preprorenin, an aliquot of renin-mRNA-enriched fraction was translated and immunoprecipitated. Molecular weight markers were [¹⁴C]methylated myosin (200,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

Fig. 2 Partial restriction enzyme map of the cloned cDNA fragment. The location of *Hae* III, *Hinf* I, *Sau* 3A and *Pst* I sites is presented. Restriction enzymes, *Eco* RI, *Bam* HI, *Hind* III, *Sal* I, *Bgl* II, *Hinc* II did not cleave the insert. The open box indicates the renin cDNA fragment. bp, base pairs

used as a template for cDNA synthesis.

Selection of Clones Carrying Renin cDNA Inserts

Bacterial clones containing renin cDNA-pBR322 recombinants were identified by the positive hybrid selection assay. Plasmid DNAs from the 106 antibiotic-selected clones were bound to nitrocellulose paper and hybridized with poly(A)⁺RNA extracted from the submandibular glands of male mice. The RNA hybridizing with the plasmid DNA was eluted and translated in a reticulocyte lysate system and the labeled products were analyzed by immunoprecipitation and SDS-PAGE. Such hybridization-selection and cell-free translation of the mRNAs identified clone 49 as a clone containing a renin cDNA insert (Fig. 1, Lane 4).

Sizing and Partial Restriction Mapping of the cDNA Insert

The cDNA insert was cleaved away from the vector pBR322 by restriction endonuclease *Pst* I and sized on polyacrylamide gels. The *Pst* I digestion resulted in a single insert fragment indicating the absence of a *Pst* I site in the cloned cDNA sequence. The insert is approximately 700 base pairs in size as determined by comparison with known *M_r* markers.

Locations of other restriction sites were determined using the recombinant plasmid (pMSR 49). Partial digestion of this fragment with several restriction enzymes enabled construction of the map shown in Fig. 2.

Detection of Renin Specific Sequences in Restriction Fragments of Genomic DNA

Renin gene was localized on DNA fragments from various sources using the Southern transfer

technique and nick-translated renin cDNA. Digestion of total chromosomal DNAs of mouse, rat, calf, and human with the endonuclease *Eco*RI, followed by electrophoretic separation on an agarose gel, transfer to a

Fig. 3 Cross hybridization of the cloned renin cDNA and total genomic DNA from different species. Total genomic DNAs were extracted from mouse (Lane 1), rat (Lane 2), calf (Lane 3) and human (Lane 4), digested with *Eco* RI restriction endonuclease, size-fractionated by 1% agarose gel electrophoresis (20 μ g per lane), transferred to nitrocellulose, hybridized with ³²P-labelled mouse submandibular gland renin cDNA, and visualized by autoradiography. λ DNA cleaved with *Hind* III was electrophoresed on the same gel and stained with ethidium bromide for use as a molecular weight marker.

nitrocellulose filter, and hybridization to nick-translated ^{32}P probe yielded a simple blot pattern for all samples examined (Fig. 3).

Cross hybridization of the mouse submandibular renin cDNA with various mammalian genomic DNA indicates that there is a high sequence homology among the renin genes of animals including human.

DISCUSSION

Panthier *et al.* (9) have determined very recently the complete amino acid sequence of mouse submandibular gland renin by nucleotide sequence analysis of their renin cDNAs. The cutting sites of endonucleases on renin cDNA prepared in the present study (Fig. 2) coincide completely with those predicted from the nucleotide sequence reported by Panthier *et al.* (9). The sequence comparison also revealed that our cDNA contains a coding region of 700 nucleotides which specify about 170 COOH-terminal amino acids of the 337 amino acid sequence of mouse submandibular renin (9).

In the present study, we have constructed and analyzed a cDNA clone containing a large portion of renin sequence. This cDNA clone, which cross-hybridizes with human renin gene, will be a valuable probe in studying the steady state levels of mRNA and the organization and expression of renin genes of humans as well as mice.

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