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CLONING OF A cDNA COMPLEMENTARY TO RAT PREPUTIAL GLAND β-GLUCURONIDASE mRNA

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Messenger RNA was isolated from rat preputial glands by guanidine HCl extraction, ethanol and salt precipitation, followed by chromatography on oligo(dT) cellulose. Double-stranded cDNA was synthesized from the mRNA and inserted into the Pst 1 site of the plasmid pBR322 by the poly(dG)·poly(dC) tailing and annealing procedure. The hybrid plasmids were used to transform E. coli HB101. Recombinant clones were screened for those containing cDNA inserts complementary to β-glucuronidase mRNA by a hybridization-selection procedure. One clone, containing an insert of about 1.2 kilobases, hybridized to preputial gland mRNA which, when translated in vitro, gave a product that migrated with the β-glucuronidase subunit on polyacrylamide gels.

The enzyme, β-glucuronidase, is an acid hydrolase present in lysosomes of most tissues of the body. Substrates for the enzyme include β-glucuronide linkages in mucopolysaccharides and other natural glucuronides such as steroid hormones (1). A deficiency of this enzyme is responsible for the inherited lysosomal storage disorder of humans known as mucopolysaccharidosis type VII (2,3). β-glucuronidase isolated from a variety of sources, including mouse liver and kidney, rat liver and preputial gland, bovine liver, and human liver and placenta, appears to be a tetrameric glycoprotein composed of identical subunits. In addition to its lysosomal location in many tissues, the enzyme is also found in the endoplasmic reticulum of certain tissues, including liver and kidney. In mouse, the enzymes located in these two different intracellular locations appear to be synthesized from the same structural gene (1). Extensive studies on quantitative variations of enzyme levels in inbred mice have allowed mapping of the structural gene for β-glucuronidase and several regulatory loci affecting transcription of the gene (1).

The richest known source of β-glucuronidase is the preputial gland of the female rat (4). The rat preputial gland enzyme has structural and biochemical properties similar to the mouse liver enzyme, and like the mouse enzyme, its level is influenced by the level of specific hormones (5,6).

Abbreviations used: EDTA, ethylene diaminetetraacetic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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Although β-glucuronidase is probably the most thoroughly studied of the acid hydrolases, many questions remain concerning unique features of β-glucuronidase gene expression and the role of the enzyme in its extralysosomal locations. The fine structure of the gene for the enzyme and that of genomic elements which may regulate its expression have not been investigated. The amino acid sequence of the enzyme subunit is also unknown. The availability of a cDNA probe containing sequences complementary to β-glucuronidase mRNA would permit selection and characterization of β-glucuronidase genomic DNA and analysis of the regulation of enzyme synthesis, as well as suggesting the amino acid sequence of the enzyme's polypeptide subunits.

This paper describes the synthesis and cloning of a cDNA containing sequences complementary to β-glucuronidase mRNA from rat preputial gland.

Materials and Methods

Preparation of cDNA. Messenger RNA was isolated from preputial glands of sexually mature female rats by initial homogenization of the minced tissue in 20 mM sodium acetate, pH 5.0 containing 6 mM guanidine HCl and 1 mM dithiothreitol followed by the subsequent purification steps of Strohman, et. al. (7). After RNA precipitation from 3 M sodium acetate, poly(A) RNA was separated from total cellular RNA by chromatography on an oligo(dT) cellulose column as described by Krystosek, et.al. (8). The first strand of a complementary DNA was synthesized using total poly(A) mRNA as a template for AMV reverse transcriptase (gift of J.W. Beard) and with oligo$(dT)_{14}$ (P.L.Biochemicals) as primer according to the procedure of Shapiro and Hollis (9). After incubation at 37°C for 60 min, the assay mixture was extracted with phenol-chloroform (1:1), and the extract passed through Sephadex G-100 (0.6 x 12.5 cm column) in 10 mM Tris, 1 mM EDTA, pH 7.0. The nucleic acid eluting in the void volume was precipitated with ethanol after addition of sodium acetate, pH 6.0 to 0.2M. The pellet was collected by centrifugation, the mRNA destroyed by alkaline hydrolysis in 0.1 M NaOH at 70°C for 20 min, and the double-stranded cDNA synthesized with reverse transcriptase and made blunt-ended with S_1 nuclease according to Ullrich, et. al. (10). The double-stranded cDNA was then phenol-chloroform extracted, passed through a Sephadex G-100 column (0.6 x 12.5 cm) and the nucleic acid in the void volume concentrated by ethanol precipitation.

Preparation of hybrid plasmids and transformation of E. coli. Terminal transferase (Bethesda Research Laboratories) was used to add approximately 15 dCMP residues to the 3' ends of the double-stranded cDNA (11) during a 5 min incubation at 37°C. In a similar manner, 10 dGMP residues were added to the 3' ends of Pst 1-cut pBR322 during a 10 min incubation at 37°C. The reactions were terminated by addition of EDTA to 25 mM, extracted with an equal volume of phenol-chloroform (1:1), and the extracts passed through Sephadex G-100 (0.6 x 12.5 cm column) in 0.1 M NaCl, 10 mM Tris, pH 7.5, 0.2 mM EDTA. Equimolar amounts of tailed plasmid and double-stranded cDNA were mixed, heated to 65°C for 3 min, and allowed to anneal slowly during an initial incubation for 2 hrs at 45°C followed by a slow cooling to room temperature. The plasmids were used to transform E. coli HB101. Approximately 50 ng of hybrid plasmid was added to 200 μl of calcium-treated cells (12). After incubation at 0°C for 10 min followed by incubation at 37°C for 5 min, 1 ml of L broth was added and the cells incubated at 37°C for 45 min. Aliquots of the cells (200 μl) were spread on 100 mm plates of L agar containing 10 μg/ml tetracycline. Tetracycline-resistant colonies were transferred to plates of L agar with 100 μg/ml ampicillin. Tetracycline-resistant, ampicillin-sensitive colonies were kept for further study.

Screening of recombinant colonies. Tetracycline-resistant, ampicillin-sensitive colonies were screened for recombinants by the in situ colony hybridization procedure of Grunstein and Hogness (13) using a ^{32}P-labeled probe synthesized from preputial gland mRNA. Incubation of the mRNA with 100 μCi of [^{32}P]ATP in the reaction mixture used for synthesis of the first strand of cDNA, gave a product labeled with 3 x 10^6 cpm/μg of DNA. Colonies which hybridized well with the probe were screened further.

Plasmids of interest were isolated from 3 ml cultures (14), digested with Pst 1 at a concentration of 1 unit/μg of DNA at 30°C for 2 hrs and examined by electrophoresis on agarose gels. Plasmids with larger inserts were screened by hybridization-selection for those containing material complementary to β-glucuronidase mRNA by the method of Ricciardi, et.al. (15). Clones to be tested were grown in M9 medium containing 10 μg/ml tetracycline. After treatment of the culture with chloramphenicol at 170 μg/ml to amplify the plasmid DNA, plasmids were isolated from lysates of the cultures (16) by banding in a cesium chloride gradient containing ethidium bromide. Plasmid DNA (1-2μg) was treated with Pst 1 to release the insert, denatured in a boiling water bath for 30 sec, and transferred to a nitrocellulose paper disc. Hybridization of total preputial gland RNA (30-40 μg) to the nitrocellulose-bound DNA was carried out in the presence of 65% deionized formamide at 50°C for 2 hrs. The hybridized RNA was eluted from the paper and concentrated by ethanol precipitation.

Translation of mRNA and immunoprecipitation. The selected mRNA was used to direct cell-free protein synthesis in a rabbit reticulocyte lysate system (Bethesda Research Laboratories) with either added [^{3}H]-leucine or [^{35}S]-methionine. Control mixtures contained either no messenger RNA or a rabbit globin messenger RNA provided with the rabbit lysate system. At the end of a 45 min incubation period at 37°C, aliquots of the reaction mixture were taken for determination of total protein synthesis as measured by counts per minute precipitable in cold 10% trichloroacetic acid, and for protein immunoprecipitable by β-glucuronidase antiserum. Immunoprecipitations were done in a manner similar to that of Palmiter (17) with the addition of 20 μg of unlabeled β-glucuronidase as carrier to the initial mixture, followed by addition of 200 μl of anti-β-glucuronidase serum, a concentration slightly in excess of that needed to precipitate the added enzyme. Analysis of [^{35}S]-methionine labeled in vitro translation products was carried out by electrophoresis on 0.1% SDS-7.5% polyacrylamide gels. Proteins were located by staining the gels with Coomassie Brilliant Blue R-250 and labeled compounds were located by autoradiography.

Preparation of β-glucuronidase and β-glucuronidase antiserum. The enzyme was purified from female rat preputial glands by the procedure of Tulsiani, et. al. (4), through the step of DE52 column chromatography. Only one protein-staining band was observed when 20 μg of the purified enzyme was analyzed on SDS-polyacrylamide gels. Antisera were prepared to the enzyme by subcutaneous injection of a rabbit at weekly intervals until the titer of precipitating antibody reached a maximum level. Injections consisted of 150 μg of enzyme in 1 ml of phosphate buffered saline emulsified with an equal volume of Freund's adjuvant, complete for the first injection and incomplete thereafter. The rabbit was bled and the antisera stored at -70°C. The antiserum gave a single precipitin line on Ouchterlony double diffusion plates when tested against the purified enzyme.

Biosafety precautions. Construction and analysis of recombinant plasmids were carried out in compliance with NIH Guidelines for Recombinant DNA Research.

Results and Discussion

The preputial gland of the female rat is the richest known source of β-glucuronidase, with the enzyme reported to compose about 7% of the gland's protein (4). If one assumes that the amount of β-glucuronidase reflects the amount of β-glucuronidase mRNA, then the gland should be an excellent source for isolation of mRNA

TABLE I

Translation and Immunoprecipitation of Preputial Gland mRNA*

Addition	TCA Precipitable cpm x 10^{-3}	Immuno-precipitable cpm x 10^{-3}	$\frac{\text{Immunoprecipitable cpm}}{\text{TCA precipitable cpm}} \times 100$
Rabbit globin mRNA 0.1 μg	119	1.6	1.3
Rat preputial gland mRNA			
Preparation A 8 ng	123	13.0	10.6
Preparation B 3 ng	35	5.2	14.8

*The assay protocol is that recommended by Bethesda Research Laboratories with conditions of incubation time, and K^+ and Mg^+ concentrations optimized for the rat mRNA. Incubation mixtures of 120 μl were used. The incorporation of [^{3}H] leucine into TCA precipitable material is assayed by a filter trapping method. The immunoprecipitation method is described in the Methods section. Blank values (no mRNA addition) of 40,000 cpm for the total TCA precipitable counts and 1,675 cpm for the immunoprecipitable counts have been subtracted from the experimental values.

to prepare a β-glucuronidase cDNA clone. To determine the amount of β-glucuronidase mRNA in the gland, total gland mRNA was used to stimulate [^{3}H]-leucine incorporation into acid-precipitable material in a rabbit reticulocyte system and the amount of labeled product immunoprecipitated by antiserum to β-glucuronidase was measured. Table 1 shows the amount of incorporation directed by preputial gland mRNA as compared with control rabbit globin mRNA. For two different preparations of rat mRNA approximately 11% and 15% of the labeled product was precipitated by β-glucuronidase antiserum as compared with 1.3% of product from a rabbit globin mRNA incubation. The latter figure may represent the experimental error of the method.

Complementary DNA was synthesized using total poly(A) mRNA from rat preputial gland as a template for reverse transcriptase. Electrophoresis of the blunt-ended double-stranded cDNA on 1% agarose gels showed that most of the labeled material migrated in the 0.9 to 1.5 kilobase range. Terminal transferase was used to add approximately 15 dCMP residues to the double-stranded cDNA and 10 dGMP residues to pBR322 which had been made linear with Pst 1. After annealing of the tailed molecules, the recombinant plasmids were used to transform E. coli HB101. Forty-six tetracycline-resistant, ampicillin-sensitive colonies were isolated to give a transformation frequency of 1 x 10^3 transformants per μg of the tailed double-stranded cDNA. Twenty-two of these colonies hybridized well to a ^{32}P-labeled cDNA probe synthesized from the original mRNA by reverse transcriptase.

Plasmid DNA was isolated from small cultures of the colonies of interest, treated with Pst 1, and examined by electrophoresis to determine the size of the

TABLE II

Translation of Hybridization Selected mRNA*

Addition	TCA Precipitable cpm x 10^{-2}	Immuno-precipitable cpm x 10^{-2}	$\frac{\text{Immunoprecipitable cpm}}{\text{TCA precipitable cpm}} \times 100$
Preputial gland mRNA	1452	125	8.6
pBR322	0	0	0
N31	67	60	89.6
K50	65	0	0
M2	177	3	1.7

*Assay conditions are as described in the Methods section and in the footnote to Table I except that incubation mixtures of 80 μl were used. Blank values (no mRNA addition) of 8400 cpm for the total TCA precipitable counts and 200 cpm for the immunoprecipitable counts have been subtracted from the experimental values.

DNA inserts. Although some of the recombinants did not have both Pst 1 sites regenerated as indicated by the fact that the inserts were not released by Pst 1 digestion, several had excisable inserts of 0.8 to 1.3 kilobases which correlated well with the size of the cDNA used for tailing.

To detect plasmids with inserts complementary to β-glucuronidase mRNA, the hybrizidation-selection procedure of Ricciardi, et. al. (15) was used. Representative results are shown in Table II. The control mRNA which was incubated in the formamide hybridization solution, when precipitated and translated, stimulated significant incorporation of [^{3}H]-leucine into acid precipitable material. About 8.6% of the labeled material was immunoprecipitated by β-glucuronidase antiserum. While the mRNA selected by several clones also stimulated [^{3}H]-leucine incorporation into an acid precipitable product, only the clone N31 selected mRNA which directed the synthesis of a labeled protein that was nearly 90% immunoprecipitable by β-glucuronidase antiserum.

Translated products and immunoprecipitated proteins from [^{35}S]-methionine labeled in vitro translations of total mRNA and of mRNA selected by N31 DNA were analyzed by electrophoresis on SDS-polyacrylamide gels (Figure 1). Lanes 2 and 4 represent total translated products from N31 selected mRNA and from total mRNA respectively. Lanes 1 and 3 show labeled materials from the same samples that were immunoprecipitated by β-glucuronidase antiserum. Lanes 1 through 4 each show a band which migrates in a manner similar to the β-glucuronidase subunit.

Figure 2 shows the migration of plasmid N31 DNA on a 1% agarose gel before and after digestion with the restriction endonucleases Pst 1 and Eco R1. The

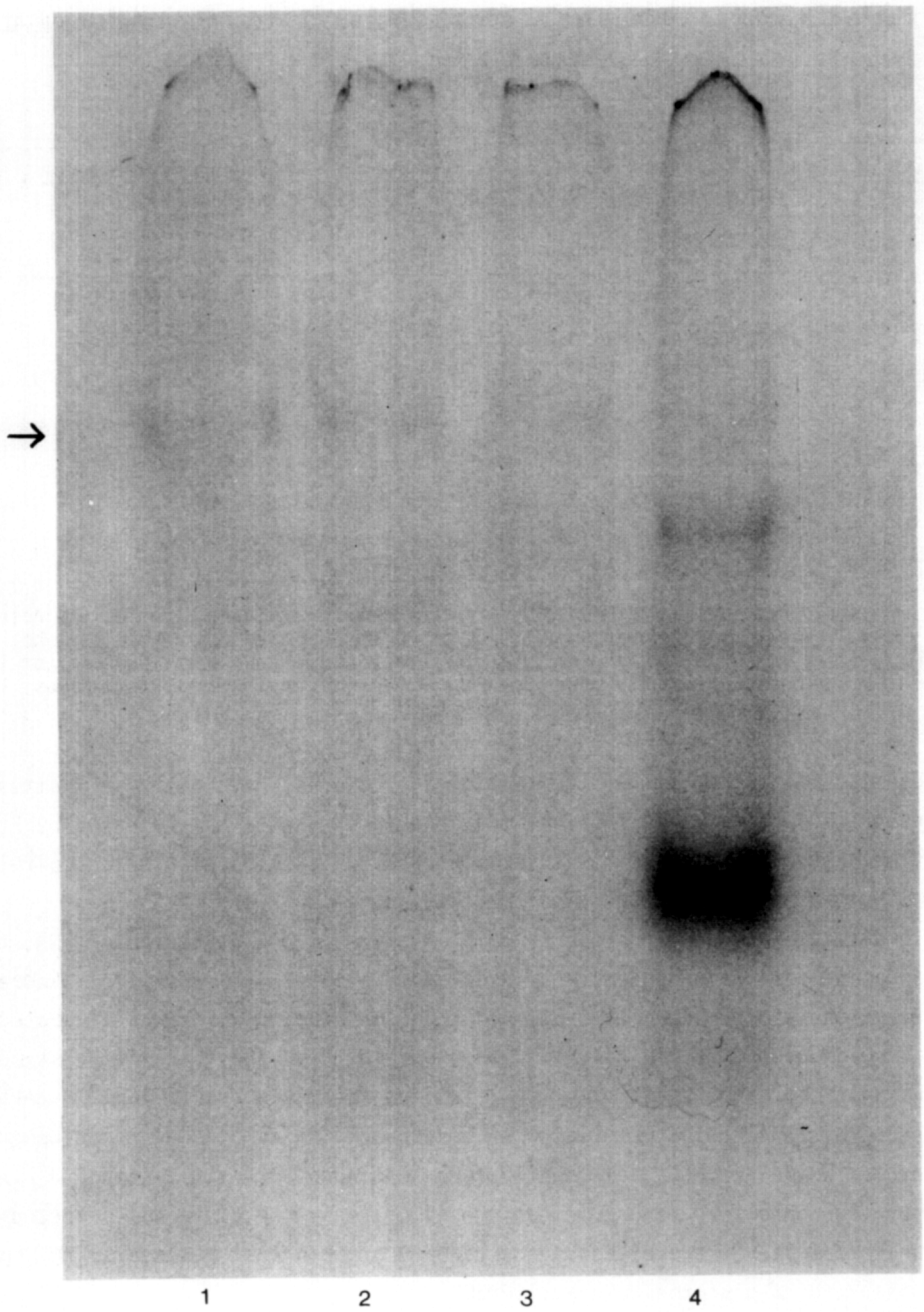

Fig. 1. Polyacrylamide gel electrophoresis of translation products and immunoprecipitated proteins from in vitro translations of total preputial gland mRNA and of mRNA selected by N31 DNA. Lanes 2 and 4, total in vitro translated products from N31 selected mRNA and from total mRNA, respectively. Lanes 1 and 3, immunoprecipitated products from in vitro translations of N31 selected mRNA and total mRNA. Arrow indicates mobility of the subunit of β-glucuronidase.

inserted fragment was released upon treatment with Pst 1 giving a fragment which migrates close to the 1.353 kilobase fragment of Hae III digested ϕX174 as well as a fragment which migrates with Pst 1 digested pBR322. Treatment of

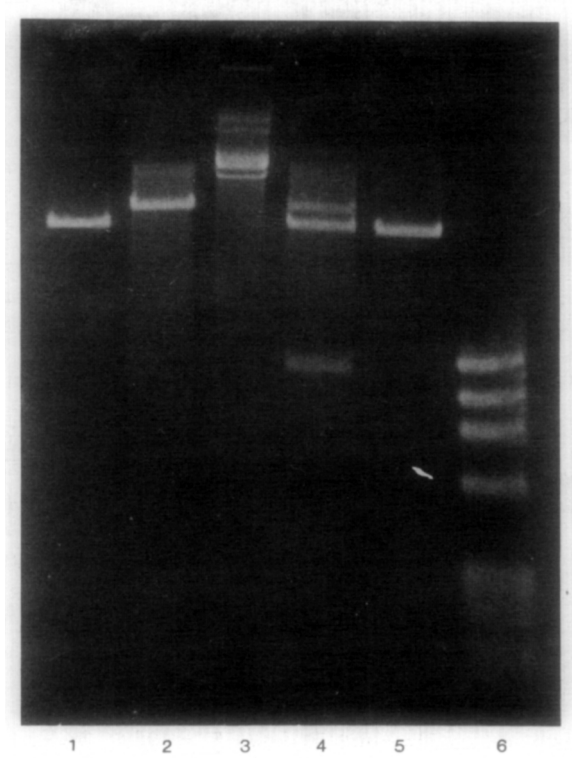

Fig. 2. Digestion with restriction endonucleases followed by agarose gel electrophoresis. Lane 1, Eco RI digest of pBR322; lane 2, Eco RI digest of N31; lane 3, undigested N31; lane 4, Pst I digest of N31; lane 5, Pst I digest of pBR322; lane 6, Hae III digest of ϕX174 with clearly resolved fragments of 1.353, 1.078, 0.872, and 0.603 kilobases.

the plasmid with Eco RI yielded only one band of DNA migrating more slowly than Eco RI digested pBR322. Thus the insert apparently contains no Eco RI sites.

This is the first report on cloning a cDNA complementary to β-glucuronidase mRNA. The availability of this clone now makes a number of interesting studies feasible. The clone can be used to select similar genomic sequences from cloned rat DNA. From a comparison of the nucleotide sequences of the cloned cDNA and complementary cloned genomic DNA, it should be possible to define the structural gene, inserted sequences, and transcriptional control regions in the genomic DNA, and to infer the amino acid sequence of the enzyme's polypeptide subunit. The rat cDNA clone can also be used to select genomic clones from mouse and human DNA libraries for studies on evolution of β-glucuronidase gene structure, hormonal regulation of β-glucuronidase gene expression, and defects involved in patients with Type VII mucopolysaccharidosis at the molecular level.

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References

1. Paigen, K. (1979) Ann. Rev. Genet. 13, 417-466.
2. Sly, W.S., Quinton, B.A., McAlister, W.H., and Rimoin D.L. (1973) J. Pediatr. 82, 249-257.
3. Hall, C.W., Cantz, M., and Neufeld, E.F. (1973) Arch. Biochem. Biophys. 155, 32-38.
4. Tulsiani, D.R.P., Keller, R.K., and Touster, O. (1975) J. Biol. Chem. 250, 4770-4776.
5. Beyler, A.L., and Szego, C.M. (1951) Am. J. Physiol. 167, 768.
6. Beyler, A.L., and Szego, C.M. (1954) Endocrinol. 54, 334-337.
7. Strohman, R.C., Moss, P.S., Micou-Eastwood, J., and Spector, D. (1977) Cell 10, 265-273.
8. Krystosek, A., Cawthon, M.L., and Kabat, D. (1975) J. Biol. Chem. 250, 6077-6084.
9. Shapiro, D.J., and Hollis, J.B. (1977) J. Biol. Chem. 252, 5244-5250.
10. Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischer, E., Rutter, W.J., and Goodman, H.M. (1977) Science, 196, 1313-1319.
11. Roychoudhury, R., Jay, H., and Wu, R. (1976) Nucleic Acids Res. 3, 863-877.
12. Wensink, P.C., Finnegan, D.J., Donelson, J.E., and Hogness, D.S. (1974) Cell 3, 315-325.
13. Grunstein, M., and Hogness, D.S. (1975) Proc. Natl. Acad. Sci. USA 72, 3961-3965.
14. Holmes, D.S., and Quigley, M. (1981) Analytical Biochem. 114, 193-197.
15. Ricciardi, R.P., Miller, J.S., and Roberts, B.E. (1979) Proc. Natl. Acad. Sci. USA 76, 4927-4931.
16. Guerry, P., LeBlanc, D.J., and Falkow, S. (1973) J. Bacteriol. 116, 1064-1066.
17. Palmiter, R.D. (1973) J. Biol. Chem. 240, 2095-2106.