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mRNA-directed synthesis of catalytically active mouse β-glucuronidase in *Xenopus* oocytes

[poly(A)-containing RNA/mouse kidney/heat-stable enzyme/antibody affinity chromatography/radioisotope incorporation]

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ABSTRACT **Catalytically active mouse β-glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31) is formed when *Xenopus* oocytes are injected with mouse RNA enriched for poly(A)-containing mRNA sequences. With the RNA from androgen-induced kidneys, the efficiency of translation is comparable to that of endogenous *Xenopus* messenger, and the fidelity of translation is high. Detection of glucuronidase messenger by formation of a catalytically active product is several orders of magnitude more sensitive than detection by incorporation of isotopically labeled amino acids. As well as providing a sensitive technique for examining the regulation of gene expression, the system makes available an opportunity to study the regulation of post-translational polypeptide processing of a lysosomal enzyme.**

A central problem in mammalian genetics is the need to define genetic regulatory elements at a molecular level of function, especially in terms of their ability to modulate the concentration of messenger RNA. A major limitation has been the low sensitivity of mRNA assays that only permit detection of relatively abundant species of mRNA. A messenger assay is particularly desirable for the system of murine glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31), one of the better characterized genetic systems of mammals (reviewed in ref. 1). Several alleles are known for the structural gene of this enzyme and two specific regulatory elements have been described that map in close proximity to the structural gene. One controls response of the structural gene to induction by androgens and the other determines the developmental program for enzyme synthesis as a function of cell type and stage of development. Additional unlinked loci are known, which determine the presence of the androgen receptor required for enzyme induction and the intracellular location of the enzyme.

Because a highly sensitive fluorometric assay for this enzyme is available, we were prompted to test whether its mRNA can direct the synthesis of a catalytically active polypeptide product. For a source of mRNA we chose androgen-induced mouse kidney and, as an appropriate assay system, *Xenopus* oocytes. As developed by Gurdon and colleagues (2), oocytes appeared the most likely system capable of synthesizing a native reaction product, as well as providing high translation efficiency and mRNA stability.

We now report that *Xenopus* oocytes do synthesize catalytically active murine β-glucuronidase in response to injection of mouse mRNA. The synthesized enzyme can be detected by its catalytic activity at several orders of magnitude lower concentration than is possible by isotope incorporation methods. This increase in sensitivity makes it possible to assay an mRNA species that is only present in the moderate to least-abundant frequency class (3).

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We have no reason to believe that similar assays cannot be developed for other mRNA species of interest.

MATERIALS AND METHODS

Isolation of Mouse Kidney RNA. Female mice of the A/J strain, 10–18 weeks old, were obtained from The Jackson Laboratory. To induce high levels of kidney β-glucuronidase activity, pellets containing about 30 mg of testosterone (generously provided by the Schering Corp.) were implanted subcutaneously 3 weeks or more before the experiment. Mice were killed by cervical dislocation and poly(A)-containing RNA was prepared from kidney polysomes or from kidney RNA obtained by guanidine extraction. Polysomes were prepared by homogenizing the tissue in a Dounce glass homogenizer in 8 volumes of cold polysome buffer (25 mM Tris·HCl/25 mM NaCl/5 mM $MgCl_2$/250 mM sucrose), pH 7.5, containing heparin at 100 μg/ml, spermine at 50 μg/ml, and mercaptoethanol at 5 mg/ml. The homogenate was centrifuged for 10 min at 12,000 × *g* maximum, and the supernatant was decanted, made 1% in Nonidet-P40 detergent (from Particle Data Laboratories), layered over 1.7 ml of 1 M sucrose in polysome buffer, and centrifuged at 48,000 rpm for 90 min in a Spinco 50Ti rotor. The polysome-containing pellets were resuspended in 0.5 M NaCl/0.5% sodium dodecyl sulfate at room temperature and fractionated over a column prepared with 0.5 g of type T-2 oligo(dT)-cellulose (from Collaborative Research). Poly(A)-containing RNA was eluted with water.

Guanidine extraction of kidney RNA followed the method of Cox (4). Kidneys were homogenized in a Dounce glass homogenizer in 8–10 volumes of cold 8 M guanidine buffered at pH 7 with 0.01 M EDTA. Nucleic acids were precipitated from this homogenate with 0.5 volume of ethanol at −10°. The precipitate was then resuspended in 4 M guanidine/0.01 M EDTA, pH 7.0, and precipitated with 0.5 volume of ethanol in the cold, and this process was repeated four times. RNA obtained by successive extractions of the guanidine/ethanol pellet with water was fractionated over an oligo(dT)-cellulose column.

Translation of mRNA in Frog Oocytes. *Xenopus laevis* mature females were purchased from Mogul-ED, Oshkosh, WI. Frogs were cooled down in ice water and killed by pithing, the ovaries were removed, and large oocytes (1–1.3 mm) were separated for injection. In a typical experiment about 30 nl of a water solution of RNA enriched for poly(A)-containing mRNA was injected per oocyte. Some controls were injected with an equivalent volume of water. Batches of 10 oocytes were incubated at room temperature in 100 μl of modified Barth's medium (2) containing 50 μCi of [^{3}H]leucine (from Amersham/Searle) having a specific activity of 10–50 Ci/mmol. After incubation the medium was removed and the oocytes were homogenized in groups of 10 to 50 oocytes per ml of 0.15 M NaCl/0.1 M Tris buffer, pH 7.5, containing 1% (vol/vol)

Triton X-100. Homogenization was performed in a small test tube by stirring the tube in a vortex mixer while holding a glass rod inside. The homogenate was then sonicated in a sonicator (Heat Systems-Ultrasonics, model w-185) with a microtip for 2 sec at 30 W and centrifuged for 30 min at 4000 × *g*. The supernatant was used directly for enzyme activity determinations and gel electrophoresis.

β-Glucuronidase Assay. β-Glucuronidase activity in the range 0.01–1 milliunit was determined fluorometrically at 37° for periods of up to 24 hr using 4-methylumbelliferone-glucuronide as substrate (5). The enzyme assay is linear for up to 100 hr. One enzyme unit hydrolyzes 1 μmol of substrate per hr at 37°.

Affinity Chromatography of β-Glucuronidase. Affinity chromatography was conducted using 0.2-ml columns of Sepharose-bound goat antibody to mouse β-glucuronidase, prepared according to the method of Cuatrecasas (6). Homogenates were adjusted to pH 4.6 with acetic acid, heated at 56° for 30 min, and centrifuged at 48,000 rpm for 30 min in a 50 Ti rotor; the supernatant was adjusted to pH 7.5 with Tris buffer, and then passed through the affinity column. The column was washed with 6 ml of 0.15 M NaCl/0.10 M Tris buffer, pH 7.5, containing 1% Triton X-100 and adsorbed enzyme was then eluted with 8 M urea in 0.1 M Na acetate buffer, pH 4.6.

Polyacrylamide Gel Electrophoresis. Electrophoresis was done under nondenaturing conditions using a Tris/glycine system in 7% polyacrylamide gels and applying a constant 200 V/cm. β-Glucuronidase was stained by its activity with naphthol-AS-BI-glucuronide as substrate (7). Gel electrophoresis in the presence of sodium dodecyl sulfate was according to the method of Laemmli (8) at a constant current of 3 mA per tube. The gels were stained for protein with 0.25% Coomassie blue in methanol/acetic acid/water (5:1:5, vol/vol) as described by Burgess (9).

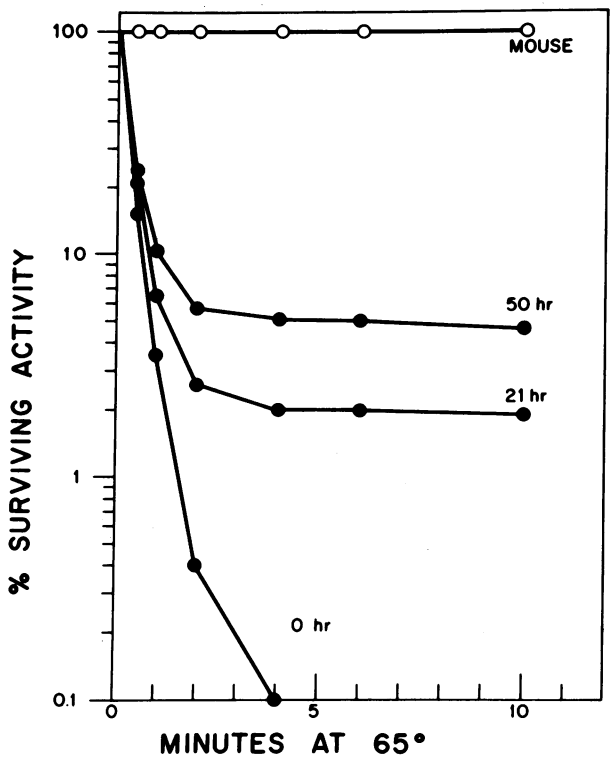

FIG. 1. Accumulation of heat-stable β-glucuronidase activity in *Xenopus* oocytes injected with mouse mRNA. Oocytes injected with 60 ng each of RNA enriched for poly(A)-containing mRNA were incubated for 0, 21, or 50 hr (●), at which time 7.7 milliunits of total glucuronidase were present per oocyte, primarily endogenous frog enzyme. Oocytes were homogenized in batches of 8. Samples containing the equivalent of 0.56 oocyte were heated at 65° in 0.1 M acetate buffer, pH 4.6, for various lengths of time and aliquots were taken for enzyme assay. Mouse kidney homogenate (○) was heated in similar manner.

RESULTS

Formation and Properties of Catalytically Active Mouse β-Glucuronidase. *Xenopus* β-glucuronidase differs from mouse β-glucuronidase in having markedly lower thermal stability and more rapid electrophoretic mobility. In addition, the frog enzyme fails to react with goat antibody to mouse β-glucuronidase. These differences make it possible to detect the synthesis of mouse β-glucuronidase in frog oocytes after injection of mouse kidney messenger RNA.

The half-life of *Xenopus* glucuronidase is about 10 min at 56°, and at 65° it inactivates very rapidly. Under the same conditions the mouse enzyme is quite stable. Frog oocytes injected with mRNA from androgen-induced mouse kidney accumulate a β-glucuronidase activity that is resistant to heating at 65°, and the level of this activity is proportional to time of incubation of the oocytes after injection (Fig. 1). The heat-resistant activity denatured at 71° with the same kinetics as authentic mouse enzyme. Similar results were obtainable using RNA obtained via polysome isolation or by direct extraction with guanidine. The guanidine procedure provided more reproducible preparations.

The new heat-stable enzyme reacts with antibody to mouse β-glucuronidase, which the frog enzyme fails to do. Passage of oocyte homogenates over anti-mouse β-glucuronidase antibody columns removed most of the heat-stable activity without significant absorption of the heat-labile activity (Fig. 2). The heat denaturation curves of the column eluates showed the presence of a small amount of β-glucuronidase activity that was not retained by the antibody columns and that had thermal stability properties intermediate between frog and mouse enzymes. This may represent heteropolymer formation between frog and mouse subunits. The mouse enzyme is a 280,000-dalton tetramer (10), as are other mammalian β-glucuronidases (11–14). It is likely that *Xenopus* β-glucuronidase is also a tetramer because the frog and mouse enzymes have similar molecular weights as estimated by the method of Ferguson (15) as modified by Hedrick and Smith (16).

After polyacrylamide gel electrophoresis under nondenaturing conditions the new enzyme synthesized in response to mouse mRNA was detected as a series of bands that stain for enzyme activity and do not migrate at the position of *Xenopus* oocyte glucuronidase (Fig. 3). Control oocytes exhibit one major band of glucuronidase activity with a second rather minor band running behind. After injection with mRNA the minor band increases in intensity and three new bands appear. The new bands are heat stable, in contrast to the endogenous bands, which are heat labile.

Radioactive Labeling of Mouse β-Glucuronidase. Formation of mouse β-glucuronidase in messenger-injected oocytes can also be detected by incorporation of radioactive amino acids, confirming that it represents the product of *de novo* protein synthesis. However, the mouse enzyme is far more difficult to detect by this method than by its catalytic activity. Large batches of injected and control oocytes were incubated in medium containing [^{3}H]leucine and then homogenized and heat treated at 65° for 10 min. The mouse β-glucuronidase present was purified by affinity chromatography on an anti-

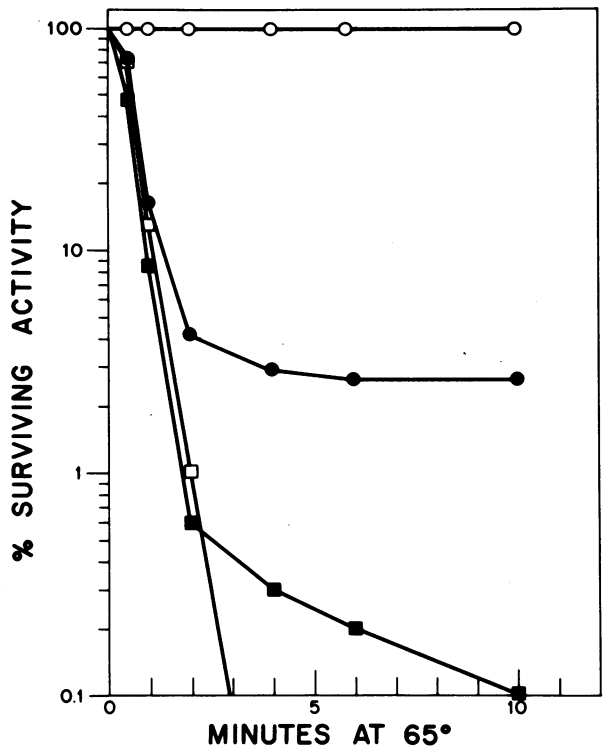

FIG. 2. Adsorption of heat-stable β-glucuronidase by antibody to the mouse enzyme. Oocytes injected with 28 ng each of RNA enriched for poly(A)-containing mRNA were incubated 4 days and then homogenized. Heat denaturation was carried out on injected oocyte homogenates before (●) and after (■) passage over anti-mouse β-glucuronidase antibody-Sepharose columns, and on homogenates of uninjected oocytes (□) and mouse kidney (○).

mouse-β-glucuronidase antibody column and subjected to electrophoresis in sodium dodecyl sulfate/polyacrylamide gels (Fig. 4). A single major peak was present, accounting for 0.03% of the label incorporated into trichloroacetic acid-precipitatable material. The radioactive product in mRNA-injected oocytes seems to be slightly larger than the β-glucuronidase subunit found in the authentic mouse L form β-glucuronidase marker used.

DISCUSSION

When injected with mouse mRNA, *Xenopus* oocytes synthesize a new form of catalytically active glucuronidase. The new enzyme reacts with anti-mouse glucuronidase antibody, which frog enzyme does not; and, like authentic mouse enzyme, the new protein denatures at 71°, rather than at 56° characteristic of frog enzyme. There is a concomitant incorporation of radiolabeled amino acids into the immunologically isolated product.

The sensitivity of detecting mRNA by its ability to direct the synthesis of a catalytically active product is very great. The injection of 30 ng of RNA enriched for poly(A)-containing sequences from induced kidney, where glucuronidase comprises 0.3% of the total protein synthesis, produces approximately 200 microunits of enzyme per oocyte per day for several days. Because the limit of assay is approximately one microunit, it is apparent that very small amounts of mRNA can be detected.

The relative efficiency and fidelity of translation of the injected mRNA is high. In induced kidney glucuronidase mRNA accounts for approximately 0.3% of the amino acid incorporation. After injection into oocytes approximately 0.03% of the

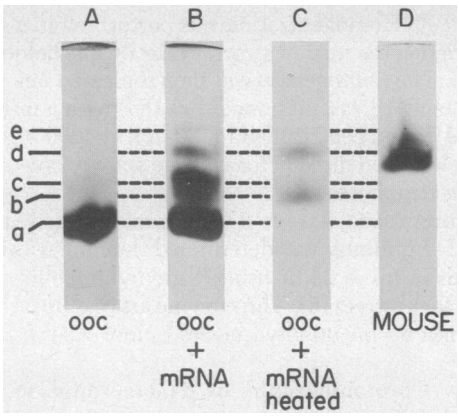

FIG. 3. Electrophoresis of mouse mRNA-directed β-glucuronidase. Gels run under nondenaturing conditions were stained for β-glucuronidase activity. Samples containing 40 units were: A, uninjected oocytes; B, mRNA-injected oocytes incubated 96 hr; C, sample B heated at 65°, pH 4.6, for 10 min; D, purified L form mouse β-glucuronidase.

amino acid incorporation was into mouse glucuronidase. Allowing for dilution with endogenous frog mRNA, it appears that the frog protein-synthesizing system utilized mouse mRNA with comparable efficiency to endogenous mRNA. The isotope incorporation data suggest that many, possibly all, of the newly synthesized molecules are catalytically active. The catalytic activity of purified native mouse β-glucuronidase is 1.7 units/μg (A. J. Lusis, unpublished data). From the enzyme activity present in the radioisotope experiment of Fig. 4 we calculate that 300 pg of enzyme was made per oocyte per day. From the specific activity of the leucine supplied, the amino acid composition of glucuronidase (10), and the amount of radioactivity present in the β-glucuronidase peak of this experiment, we calculate that about 25 pg of glucuronidase was synthesized per oocyte per day if no allowance is made for dilution by the endogenous leucine pool. If the pool dilution factor is not appreciably greater than 12, then nearly all of the glucuronidase molecules synthesized were catalytically active and the fidelity of translation was high.

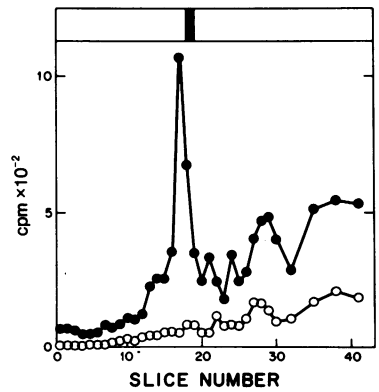

FIG. 4. Incorporation of radioactivity into mouse β-glucuronidase. A total of 100 oocytes injected with 100 ng each of RNA enriched for poly(A)-containing mRNA was incubated 48 hr in [^{3}H]leucine medium and homogenized. Incorporation into acid-insoluble material was 9.95×10^5 cpm/oocyte. β-Glucuronidase was adsorbed to antibody columns and 4540 cpm per oocyte was eluted. Eluted material equivalent to six oocytes was electrophoresed under denaturing conditions and the gels were sliced and their radioactivities were measured. Injected oocytes (●); uninjected controls (○). Above on the same scale is a parallel gel containing purified mouse L form β-glucuronidase.

As isolated from a variety of species, glucuronidase is a tetrameric glycoprotein with four identical subunits. In the mouse two tetrameric forms exist that arise from the same structural gene by a post-translational modification (7). One tetramer, with slower electrophoretic mobility, form X, is present in endoplasmic reticulum, where it is complexed with a second protein, egasyn; the other tetramer, termed L, is present in lysosomes, uncomplexed (17).

The product of mouse mRNA-directed synthesis is not identical with the enzyme isolated from mouse tissues. The subunit molecular weight, as judged by sodium dodecyl sulfate gel electrophoresis, is slightly larger than that of the mouse enzyme, indicating that processing of the nascent polypeptide chain into "mature" enzyme may not be the same in frog and mouse cytoplasm. Electrophoresis under nondenaturing conditions shows the presence of three new β-glucuronidase bands (bands b, d, and e of Fig. 3) in messenger-injected oocytes that are heat stable. Band d has the mobility of X form mouse enzyme. It is not clear to what extent the novel enzyme species represent heteropolymers between mouse and frog subunits, or to what extent they are the consequence of post-translational modification of the mouse enzyme catalyzed by frog cytoplasm. Either mechanism could account for the presence of some β-glucuronidase molecules that are not retained by anti-mouse enzyme antibody columns and have intermediate rates of heat denaturation.

We expect the new system to be useful both in answering questions concerning the synthesis and processing of glucuronidase and in examining problems of gene regulation. Enzyme synthesis in a cell previously unable to synthesize that molecule should provide new information about rates of enzyme synthesis and mechanisms of intracellular location. The availability of a sensitive assay for this mRNA species will make it possible to define more precisely the regulation of its structural gene, especially with respect to hormonal induction mechanisms and the function of the temporal genetic element determining its developmental program.

Should the mRNAs for other enzymes also direct the synthesis of catalytically active products, it will make a new level of investigation possible in studying the physiology and biochemistry of mRNA synthesis, processing, and function.

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