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Properties of rat and mouse β-glucuronidase mRNA and cDNA, including evidence for sequence polymorphism and genetic regulation of mRNA levels

(Recombinant DNA; translational activity of RNA; RNA-RNA hybridization; induction by androgen; congenic mice; *Xenopus* oocyte assay)

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SUMMARY

cDNA clones containing partial sequences for β-glucuronidase (βG) were constructed from rat preputial gland RNA and identified by their ability to selectively hybridize βG mRNA. One such rat clone was used to isolate several cross-hybridizing clones from a mouse-cDNA library prepared from kidney RNA from androgen-treated animals. Together, the set of mouse clones spans about 2.0 kb of the 2.6-kb βG mRNA. Using these cDNA clones as probes, a genomic polymorphism for DNA restriction fragment size was found that proved to be genetically linked to the βG gene complex. A fragment of βG cDNA was subcloned into a vector carrying an SP6 polymerase promoter to provide a template for the in vitro synthesis of single-stranded RNA complementary to βG mRNA. This provided an extremely sensitive probe for the assay of βG mRNA sequences. Using either nick-translated cDNA or transcribed RNA as a hybridization probe, we found that mouse βG RNA levels are strongly induced by testosterone, and that induction by testosterone is pituitary-dependent. During the lag period preceding induction, during the induction period itself, and during de-induction following removal of testosterone, βG mRNA levels paralleled rates of βG synthesis previously measured by in vivo pulse-labelling experiments. Genetic variation in the extent of induction affected either the level of βG mRNA or its efficiency of translation depending on the strain of mice tested.

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Abbreviations: AMV, avian myeloblastosis virus; βG, β-glucuronidase; bp, base pairs; DTT, dithiothreitol; *Gus-s*, gene coding for β-glucuronidase; [*Gus*], gene complex containing *Gus-s* and regulatory loci; kb, kilobases or 1000 bp; SDS, sodium dodecyl sulfate; SSC, see MATERIALS AND METHODS, section **d**; u, units.

INTRODUCTION

The acid hydrolase βG has served as a model enzyme both in the study of lysosomal enzyme storage diseases and for the investigation of mammalian gene regulation. In these latter studies, the enzyme has been shown to be both developmentally and hormonally regulated in rats and mice. The

studies in mice have been greatly facilitated by the identification of both structural and regulatory gene variants among inbred and feral strains of mice, and analysis of these variants has provided much of our understanding of how the enzyme is regulated.

A combination of genetic and biochemical techniques has shown that the structural gene for βG and a set of closely linked regulatory genes are located on mouse chromosome 5; together these are referred to as the [*Gus*] complex (for review see Paigen, 1979). Altogether, six alleles of the structural gene, *Gus-s*, are presently known. Among the regulatory loci the *Gus-r* locus acts *cis* to regulate the androgen induction of βG in mouse kidney; a temporal locus, *Gus-t*, acts *trans* to regulate tissue- and time-specific changes in βG levels; and a systemic locus, *Gus-u*, has a uniform effect on βG levels in all tissues.

To approach questions about the location of the regulatory sequences and the molecular basis of their function, and to study the organization of the βG structural gene itself, requires obtaining both mRNA and genomic sequences of βG as recombinant DNA molecules. Because rat preputial gland has exceptionally high levels of βG enzyme and mRNA activity, we were able to isolate rat cDNA clones and then use these as probes to identify βG sequences in a mouse cDNA library.

During the course of this work βG sequences have been successfully cloned in three other laboratories. Hieber (1982) reported a rat cDNA clone generated from preputial gland RNA, but this clone was not at first made available to other laboratories. More recently Palmer et al. (1983) and Catterall and Leary (1983) have isolated cDNA clones for βG directly from mouse-cDNA libraries.

The rat clone reported here was identified by its ability to selectively hybridize an mRNA that directed the in vitro translation of βG polypeptide, which was precipitated by antibody against purified rat enzyme. The identification of the mouse βG clones was confirmed by several criteria. First, when used as a probe, the mouse cDNA clones detected a genomic DNA sequence polymorphism located at or near the [*Gus*] complex. Second, the physical and biological properties of the mouse RNA hybridizing to the cloned mouse cDNA sequences closely paralleled those previously determined for βG mRNA. And finally, the induction of this mouse RNA proved to be regulated by the *Gus-r* gene.

MATERIALS AND METHODS

(a) Animals

Mice of strains A/J, carrying the $[Gus]^A$ haplotype, and C57BL/6J, carrying the $[Gus]^B$ haplotype, were obtained from the Jackson Laboratory. Congenic strains C57BL/6J.A and C57BL/6J.H have the $[Gus]^A$ and $[Gus]^H$ haplotypes moved from their strains of origin to a C57BL/6J background (Pfister et al., 1982). These congenic strains were constructed and generously provided by Dr. Verne Chapman (Roswell Park Memorial Institute, Buffalo, NY). Female Sprague–Dawley rats were purchased from Charles River Laboratories.

Mice were induced for kidney βG by subcutaneous implantation of a 30-mg testosterone pellet. This provided a continuous high dose of testosterone over an extended period of time. To induce preputial gland βG in rats, animals were injected intramuscularly on days 1, 2, 3, 7, 8 and 9 with 20 u of pregnant mare serum in 0.1 ml saline. Rats were killed on the tenth day.

(b) RNA isolation, fractionation and translational activity

RNA was isolated by the guanidine–HCl method (Cox, 1968) as described by Labarca and Paigen (1977) or by the guanidine thiocyanate method of Chirgwin et al. (1979). Enrichment for poly(A)-containing sequences and sucrose gradient centrifugation were as described by Berger et al. (1981). Translational activity of mouse or rat βG was determined by injecting *Xenopus laevis* oocytes with RNA and measuring the accumulation of mouse or rat βG enzyme activity (Watson et al., 1981).

(c) Construction of cDNA libraries

A rat preputial gland cDNA library of 7000 clones and a mouse kidney cDNA library of 3000 clones were made as described elsewhere (Berger et al., 1981). In each case first and second strands of cDNA were made using AMV reverse transcriptase; ends were blunted using S1 nuclease, and oligo(dC) tails were added using terminal deoxynucleotidyl-transferase. Tailed cDNA was annealed with pBR322 that had oligo(dG) added to the 3′-termini

generated by cutting the plasmid with *Pst*I. Recombinant DNA was then used to transform *E. coli* cells. The rat library was constructed in JW355 cells (John Williams, Pfizer Co.). For the mouse library the method of Hanahan (1983) was used to transform DH1 cells.

(d) Selection and translation of RNA

RNA was selected by hybridization to filter-bound, recombinant, plasmid DNA following a procedure modified from Schleif and Wensink (1981). About 15 μg of denatured DNA was bound to 1 cm diameter nitrocellulose discs. Multiple filter discs were hybridized for 18 h at 42°C in 0.5 ml of a mixture containing 5 × SSC, (1 × SSC is 0.15 M NaCl, 0.015 M Na_3 · citrate, pH 7.0), 25 μg poly(A) and 0.1% SDS in 50% formamide with the addition of either 120 μg poly(A)$^+$-enriched rat preputial gland RNA or 20 μg of the same RNA enriched for βG mRNA by sucrose gradient centrifugation. After hybridization the filters were washed once in 10 ml of 1 × SSC, 0.1% SDS at room temperature and three times in 5 ml of 50% formamide, 0.5 × SSC, 0.1% SDS at 37°C for 30 min. Hybridized RNA was eluted from the filter-bound DNA, precipitated with ethanol and dissolved in 2 μl sterile water. The whole sample was then added to an in vitro reticulocyte protein-translation system using a commercial translation kit (New England Nuclear) as directed. One-third of the translation products was reserved for gel electrophoresis and two thirds was treated with antibody.

For antibody precipitation, these translation products were diluted to 200 μl containing 0.1 M Tris · HCl, pH 7.5, 0.15 M NaCl, 1.0 μg purified rat βG (a gift from Dr. Philip Stahl), and sufficient rabbit anti-rat βG serum (also from Dr. Philip Stahl) to precipitate βG quantitatively. Precipitates were allowed to form overnight at 4°C, collected by centrifugation, and washed four times. The first two washes were 1 ml Tris · HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100 and the next two were the same except that Triton X-100 was replaced by 0.1% SDS. Antibody precipitates as well as total translation products (aliquot reserved prior to addition of antibody) were dissolved in loading buffer and run on 10% polyacrylamide gels in SDS according to Laemmli (1970). Radioactivity in the gels was observed by fluorography after treating with Enhance (New England Nuclear).

(e) Construction of pSP6-G3

A 1.0-kb cDNA fragment from pGA-1 (*Hin*dIII to *Eco*RV) was subcloned into a vector carrying a bacteriophage SP6 promoter (Green et al., 1983). This required converting the *Eco*RV end of the fragment to *Eco*RI with an *Eco*RI linker (Bethesda Research Laboratories). In this subclone the *Hin*dIII site was proximal to the SP6 promoter thus providing a partial βG sequence in reverse of the normal orientation with respect to a promoter. Procedures and conditions for this construction followed those described by Maniatis et al. (1982).

(f) Transcription of pSP6-G3

SP6 RNA polymerase (Butler and Chamberlin, 1982) was a gift from Dr. Michael Chamberlin, and is specific for the SP6 promoter (Kassavetis et al., 1982). Standard in vitro transcription reactions (20 μl) were modified from Green et al. (1983) and contained: 0.5 μg pSP6-G3 DNA linearized by restricting with *Eco*RI, 40 mM Tris · HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 0.5 mM of each non-labelled ribonucleoside triphosphate, 50–500 μCi of [α-^{32}P]UTP (3000 Ci/mmol, Amersham) and sufficient cold UTP to make the final concentration 12 μM, 1.5 u RNasin/ml, and 6 u of SP6 RNA polymerase. Reactions were incubated at 37°C for 1 h followed by the addition of 0.02 u of RNase-free DNase I (Boehringer Mannheim) and incubation at room temperature for 20 min. The reaction was terminated by the addition of EDTA to 10 mM. The labelled transcript was extracted with phenol, passed over an SP-Sephadex C-50 column, and ethanol-precipitated in the presence of carrier DNA. In some earlier experiments [^{32}P]GTP was used instead of [^{32}P]UTP, but proved to be less reliable. For the experiments presented here RNA specific activities of 0.5–3 × 10^9 dpm/μg were used.

(g) Hybridization

Unless otherwise indicated normal hybridization conditions using nick-translated probes were at

65°C in 4 × SSC with 10 × Denhardt (1966) mixture, 10% dextran sulfate, 0.5 mg/ml sheared, denatured herring sperm DNA, 0.2% SDS, 0.3% (w/v) sodium pyrophosphate, 0.1 M Na_2HPO_4, pH 7.0. Nitrocellulose filters with bound nucleotide sequences were incubated with labeled probe for 12–36 h, and then washed 4 times in 2 × SSC with 0.2% SDS at 65°C for 30 min. For higher stringency the salt concentration in the washes was reduced to 0.1 × SSC, and for lower stringency (rat-mouse hybrids) the temperature of the hybridizations and the washes was reduced to 60°C. An additional, final wash in 3 mM Tris · HCl, pH 9.0 at room temperature was sometimes, but not always, used.

Hybridization conditions using in vitro transcripts of pSP6-G3 as probes were at 60°C for 36–60 h in 50% formamide, 5 × SSC, 10% dextran sulfate, 10 × Denhardt (1966) mixture, 0.5% SDS, 0.2 mg/ml denatured herring sperm DNA and 1–5 × 10^6 cpm/ml ^{32}P-labeled RNA. Hybridization filters were washed 4 times at 70°C in 0.1 × SSC, 5 mM Na_2HPO_4, pH 7.0, 0.015% sodium pyrophosphate, 0.2% SDS and 2 times at room temperature in 3 mM Tris · HCl, pH 9.0. Each wash was 30 min.

(h) Other methods

The techniques for blotting RNA from formaldehyde gels onto nitrocellulose (Maniatis et al., 1982), dot blots on nitrocellulose (Thomas, 1980), labeling DNA by nick-translation (Maniatis et al., 1975), labeling DNA by single-strand replacement using T4 DNA polymerase (O'Farrell, 1981), preparation of plasmid DNA (Tanaka and Weisblum, 1975) and isolation of insert DNA fragments by agarose gel electrophoresis (Maniatis et al., 1982) have all been described.

RESULTS

(a) *Xenopus* oocyte assay of mRNA activity

As a preliminary to cloning , some of the physical and biological properties of βG mRNA were determined utilizing the *Xenopus* oocyte assay. In this assay RNA samples are microinjected into oocytes, βG mRNA is subsequently translated in vivo into active enzyme molecules, and the newly formed enzyme assayed as a measure of the amount of mRNA injected (Labarca and Paigen, 1977). This assay was used to estimate the size of βG mRNA by assaying fractions from a sucrose gradient in which poly(A)-enriched mouse kidney RNA had been sedimented. βG mRNA activity migrated as a single peak of about 20S and, using the equation $M_r = 1550 \times S^{2.1}$ (Spirin, 1963), suggests that the size of βG mRNA is about 2.5 kb.

Using the same oocyte assay, the kinetics of induction of βG mRNA activity in androgen-stimulated mouse kidney were determined previously (Watson et al., 1981). Unlike other androgen-induced mRNAs in mouse kidney (Berger et al., 1981; 1984), βG mRNA is induced very slowly, with a half-time for induction of about 8 days. The induction plateau is not reached until 25–30 days. Additionally, this induction of βG mRNA activity does not occur in hypophysectomized animals (unpublished data). Other experiments measuring induction of enzyme synthesis show that growth hormone is the required factor (Swank et al., 1978).

(b) Rat preputial gland cDNA clone

Rat preputial gland is the richest known source of mammalian βG (Ohtsuka and Wakabayashi, 1970). Using the oocyte assay we estimated the βG mRNA frequency in normal rat preputial gland RNA to be about fivefold that in RNA obtained from the kidneys of induced mice, although the concentration varied considerably from one rat to another. Treatment of female rats with pregnant mare serum resulted in a two- to fourfold increase in βG-specific enzyme activity (u/g wet weight of tissue), and also increased the weight of the preputial glands 20% to 60%. Poly(A)$^+$ RNA from the glands of 25 treated rats was isolated and further enriched eightfold for βG mRNA by taking the peak fractions after sucrose-gradient centrifugation. This enriched RNA was used to direct cDNA synthesis and generate a cloned cDNA library by methods used before (Berger et al., 1981).

When rat preputial total RNA was used to stimulate a reticulocyte lysate translation system, in vitro synthesized βG subunits were marginally detectable (Fig. 1, lane 1). Using poly(A)$^+$ RNA or sucrose-

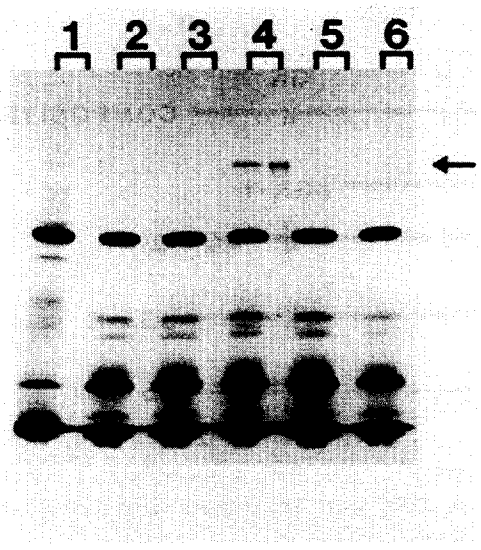

Fig. 1. Autoradiograph of in vitro translation products following SDS-polyacrylamide gel electrophoresis. Hybrid selection of RNA, in vitro translation, antibody precipitation and electrophoresis are described in M & M, section **d**. Each pair of lanes contains first the total translation products for a given RNA sample and second the immunoprecipitate of the translation products after adding anti-βG. Translated samples were: (1) rat preputial gland total RNA; (2–5) rat preputial gland RNA selected by hybridization to DNA of plasmids (2) pRP1186, (3) pBR322, (4) pRP1183, (5) pRP1188; and (6) yeast tRNA. Plasmids pRP1186 and pRP1188 contained rat cDNA inserts of unknown identity. The gel was stained for protein prior to autoradiography and the position of purified rat βG is indicated by the arrow.

gradient-enriched RNA yielded βG subunits that were readily detected by immune precipitation (not shown). To identify βG sequences in the rat cDNA library, clones were tested for their ability to selectively hybridize RNA molecules capable of directing the synthesis of βG protein in this translation system. Groups of six clones were grown together, and the plasmid DNA was isolated, denatured and bound to nitrocellulose for hybridization. Fluorographs of gels containing the translation products of hybrid-selected rat RNA indicated that three of six groups tested yielded a band of incorporated label identical in position to the subunit of authentic βG. Two of the positive groups were subdivided into individual clones and plasmid DNA was isolated and used to hybrid-select RNA as before. One of the plasmids from each group again hybridized an RNA that directed the synthesis of an immunoprecipitable polypeptide having the same M_r as the subunit for rat βG. The results for one of these plasmids, pRP1183, are shown in Fig. 1.

Using nick-translated pRP1183 as a hybridization probe, it was shown that the rat-derived clone cross-hybridized to both mouse RNA and DNA under conditions of reduced stringency. The hybridizing mouse RNA exhibited the properties previously established for mouse βG mRNA using the oocyte injection assay. It was induced by testosterone in mouse kidney over a long time course with a half-time of 7–10 days, this induction required pituitary function, and $[Gus]^A$ mice induced to a higher level of hybridizable RNA than $[Gus]^H$ mice (not shown). By Northern blot analysis the single major species of hybridizing mouse RNA had a size of 2.6 kb, which is close to the size estimated for βG mRNA using sucrose-gradient centrifugation and monitoring mRNA activity by translation in oocytes.

The insert DNA from pRP1183 is about 550 bp; it can be excised from the pBR322 vector sequence by digestion with *Pst*I, which cuts at the reconstituted insertion sites.

(c) Mouse βG cDNA clones

A mouse cDNA library was constructed from fully induced male kidney RNA of strain A/J mice, and this library was probed with purified, nick-translated insert from pRP1183. Of 2500 colonies screened, three cross-hybridized with the rat βG partial sequence. These have been designated pGA-1, pGA-2 and pGA-3, and contain insert sequences of approximately 1.45, 0.61 and 0.45 kb. A composite restriction map including two of these overlapping sequences is shown in Fig. 2. Using the larger of the two *Pst*I fragments from pGA-1 to probe the same library again, an additional clone, pGA-5, extending toward the 5′-end of the gene was found. Using the same fragment of pGA-1 to probe another similarly constructed cDNA library, several additional βG clones were identified. One of these, pGA-6, extends towards the 3′ end of the gene and is the only βG clone to include a poly(dA) sequence as evidenced by its ability to hybridize labeled oligo(dT). Clones pGA-5 and pGA-6 are included in the composite map (Fig. 2). Together these clones span about 2.0 kb of cDNA, still significantly less than the 2.6 kb expected from the length of βG mRNA.

The orientation of transcription and the identity of

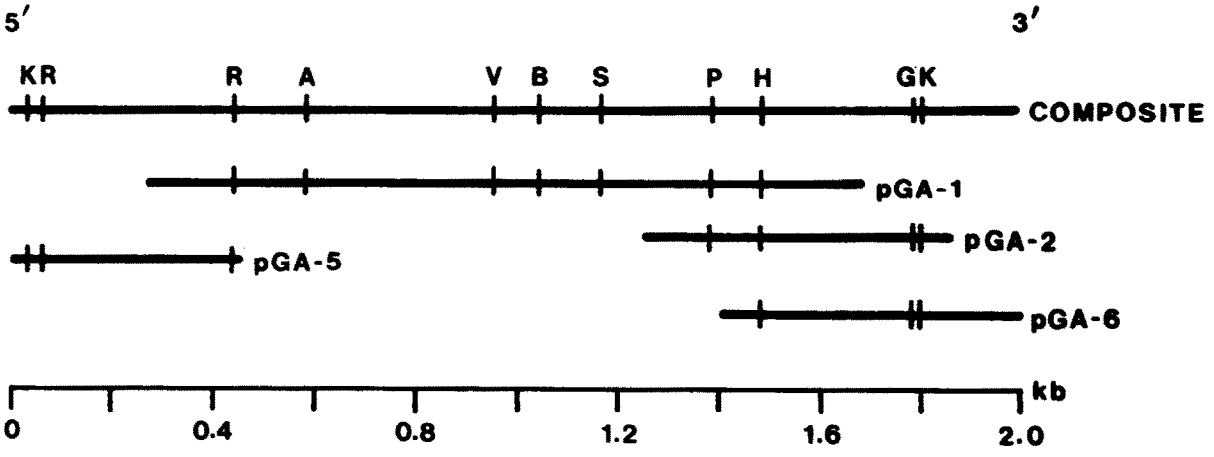

Fig. 2. Restriction map of βG cDNA clones. Restriction sites are: K, *Kpn*I; R, *Eco*RV; A, *Ava*I; V, *Pvu*II; B, *Bam*HI; S, *Sac*I; P, *Pst*I; H, *Hin*dIII; and G, *Bgl*I. Each clone has synthetic *Pst*I sites at both ends of the cDNA insert. The composite map starts 0.6 kb downstream from the 5′ end of βG mRNA and extends into the poly(A) region at the 3′ end.

the βG coding strand were determined by using strand-specific probes. Circular pGA-1 was cut at the *Sac*I site and T4 DNA polymerase was used to label opposite strands from the 3′ ends. The labeled DNA was then digested with *Pst*I and the two oppositely labeled fragments on either side of the *Sac*I site were isolated by gel electrophoresis. Only the shorter of the two fragments contained a labeled strand capable of hybridizing to induced mouse kidney RNA; therefore the left end (as drawn in Fig. 2) of the pGA-1 insert extends towards the 5′-end of the message.

(d) Restriction polymorphism

Southern blots of mouse genomic DNA probed with clone pGA-1 show a DNA fragment length polymorphism that is genetically linked to the βG gene on chromosome 5. DNA preparations from three congenic strains of mice each carrying the [*Gus*]^{A}, [*Gus*]^{B} or [*Gus*]^{H} haplotype were digested with restriction enzymes, fractionated by gel electrophoresis, blotted to nitrocellulose, and hybridized to labeled pGA-1. The results (Fig. 3) show the same pattern of *Pst*I fragments for the *B* and *H* haplotypes while the smallest fragment is missing in the *A* haplotype. Since the congenic strains were constructed by repeated backcrosses to strain C57BL/6J, 15 generations in the case of the C57BL/6J.A congenic, we can conclude that pGA-1 hybridizes to genomic DNA that is closely linked to (and probably identical with) the βG structural gene.

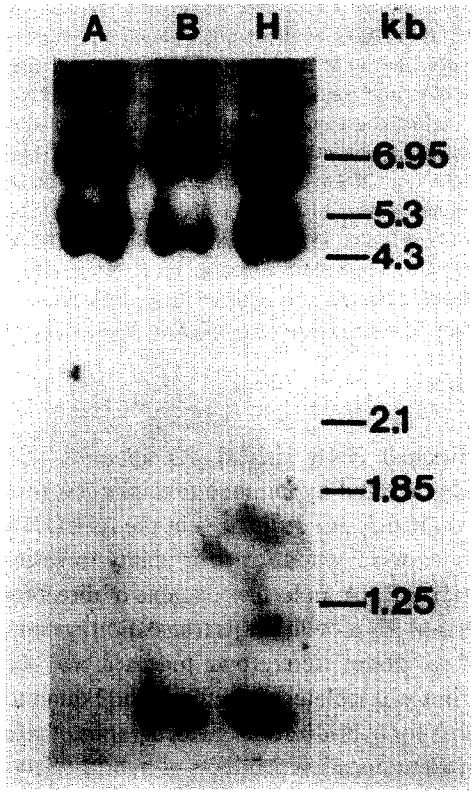

Fig. 3. Southern blot analysis of DNA sequences from different haplotypes. DNAs from congenic strains of mice carrying the A, B and H [*Gus*] haplotypes were isolated from liver nuclei as described by Piccini et al. (1982) and were digested with *Pst*I. Aliquots of 10 μg were fractionated on a 1% agarose gel, blotted to nitrocellulose (Southern, 1975), and hybridized to nick-translated [^{32}P]pGA-1 (see MATERIALS AND METHODS, section **g**). Indicated DNA fragment sizes (kb) are based on restriction fragments of λ and pBR322 DNA.

(e) Single-stranded RNA probe

To prepare a high-specific-activity single-stranded probe, the *Eco*RV-*Hin*dIII restriction fragment from pGA-1 was subcloned into a plasmid vector containing a bacteriophage SP6 promoter (Green et al., 1983) such that the *Hin*dIII end was proximal to the SP6 promoter. When transcribed in vitro using SP6-specific RNA polymerase (Butler and Chamberlin, 1982; Kassavetis et al., 1982), RNA complementary to βG mRNA was made. This confirmed the previously determined orientation of mRNA transcription shown in Fig. 2. More importantly, it provided us with a hybridization probe that was much more sensitive than nick-translated DNA.

(f) βG mRNA

Labeled RNA transcribed from pSP6-G3 hybridized to an androgen-inducible, 2.6-kb RNA in Northern blots of kidney RNA size-fractionated by gel electrophoresis (Fig. 4). A second, less prominent band of hybridizable RNA was seen at approx. 1.6 kb and was also inducible, but it corresponds to a molecular size too small to code for a complete subunit of βG. It did not represent a constant or

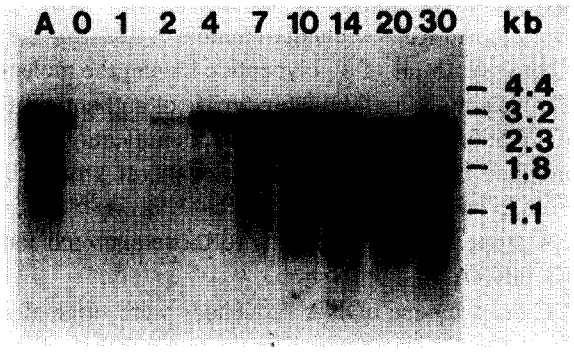

Fig. 4. Northern blot analysis of βG mRNA during induction. RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde, blotted onto nitrocellulose, and hybridized with labeled RNA (6×10^8 dpm/μg) transcribed from pSP6-G3. Lane (A) contained 3 μg poly(A)$^+$ RNA from the kidneys of fully induced A/J mice and represents an overnight exposure of X-ray film. The remaining lanes contained 15 μg total RNA from the kidneys of C57BL/6J.A congenic mice that had been administered testosterone for the number of days indicated, and represent a film exposure of 10 days. Size markers (kb) were single-stranded DNA fragments of pBR322 generated by restriction with *Pst*I (4.4), *Pst*I plus *Bam*HI (3.2 and 1.1), and *Bgl*I (2.3 and 1.8).

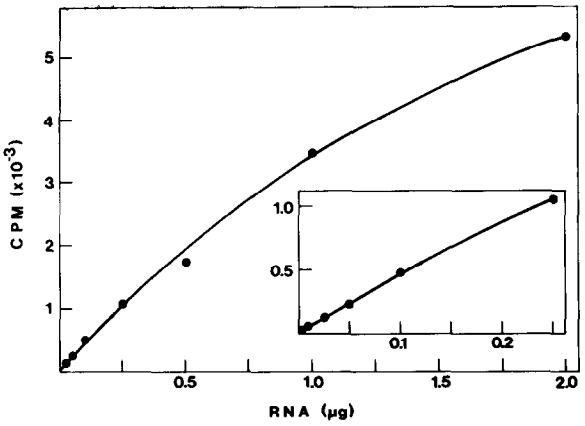

Fig. 5. Calibration of dot blot assay for βG mRNA. Total RNA from the kidneys of male A/J mice induced for 6 weeks with testosterone was diluted in water and spotted in 2-μl aliquots on nitrocellulose. After hybridization to labeled RNA (2×10^9 dpm/μg) transcribed from pSP6-G3, individual spots were cut out and hybridized ^{32}P was counted. Cpm are plotted against the amount of RNA per spot, and each point on the curve is the mean of three determinations. The inset shows in detail the lower portion of the curve.

reproducible fraction of the 2.6-kb RNA and it was diminished when RNA was enriched for poly(A)$^+$ sequences.

Dot blots containing various amounts of kidney total mRNA were hybridized with ^{32}P-labeled RNA transcribed from pSP6-G3 (Fig. 5). Hybridization was proportional to RNA for low amounts of RNA and deviated slightly from linearity at higher amounts of RNA. The extent of hybridization appeared to be determined only by the amount of βG mRNA present and was unaffected by the presence or absence of nonspecific RNA (not shown). Using probes with 3×10^9 dpm/μg RNA, we estimate that less than 2×10^5 molecules of βG mRNA must be present in a dot to double the radioactivity over background (about 40 dpm).

Included in each dot blot experiment used to determine βG mRNA concentrations was a concentration curve like that shown in Fig. 5. Thus all RNA samples could be directly compared to an equivalent amount of βG RNA in a dilution of the standard preparation of fully induced kidney RNA from mouse strain A/J.

We have quantitated βG mRNA levels in several tissues using dot blots consisting of total RNA immobilized on nitrocellulose and hybridized to labeled pGA-1 or to labeled pSP6-G3 transcripts. Of

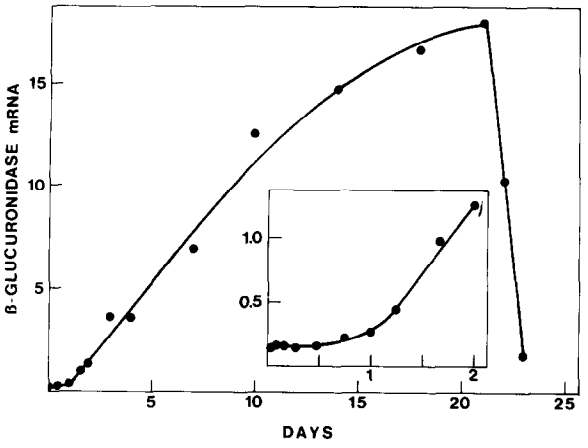

Fig. 6. Induction of βG mRNA by androgen. Testosterone pellets were implanted in female A/J mice at day zero, and kidney RNA was prepared at the times indicated. After 21 days of administration, testosterone pellets were removed and de-induction was followed for two days. Total RNA from kidneys was dot-blotted on nitrocellulose and hybridized with labeled RNA (3×10^9 dpm/μg). Levels of hybridization were determined by counting hybridized ^{32}P label and comparing to a standard curve; they are expressed as μg βG mRNA/g total RNA.

the tissues tested from androgen-treated animals (kidney, heart, liver, brain, submaxillary gland and spleen), only kidney had elevated levels of mRNA. Also, βG mRNA remained low in kidneys of hypophysectomized animals treated with testosterone, confirming the pituitary requirement for induction at the RNA level.

To measure the kinetics and the extent of induction in detail, the dot blot system probed with pSP6-G3 transcript proved particularly useful. βG mRNA concentration in the kidneys of A/J mice remained unchanged for several hours after the administration of testosterone (Fig. 6). Following this lag of about 18 h there was a long rise in concentration eventually approaching a plateau after 3 weeks. The overall extent of induction was about 120-fold. Removal of the testosterone pellets from induced females resulted in the rapid decline of βG mRNA.

We have estimated the fraction of total RNA that is βG mRNA using known amounts of intact pGA-1 as a standard and labeled insert (1.1-kb *Pst*I fragment) from pGA-1 as the hybridization probe. For fully induced kidney RNA from $[Gus]^A$ mice, the mass fraction of total RNA that corresponded to βG mRNA was 1.8×10^{-5}, or 18 μg βG mRNA/g total RNA. Assuming that mRNA is 2% of total RNA, about 9×10^{-4}, or slightly less than 0.1%, of the message is βG mRNA.

(g) Regulation of βG mRNA by *Gus-r*

Using hybridization probes to compare RNAs from the kidneys of fully induced animals indicated that $[Gus]^A$ mice had more copies of βG mRNA than $[Gus]^B$ mice (Table I). This difference was paralleled by a difference in the rates of βG synthesis, and implies that βG mRNAs from $[Gus]^A$ and $[Gus]^B$ mice are similar with respect to the efficiency of translation. Mice carrying the $[Gus]^H$ haplotype had lower rates of βG synthesis than either $[Gus]^A$ or $[Gus]^B$ mice, but had βG mRNA levels comparable to $[Gus]^B$ animals. This suggests that $[Gus]^H$ mice synthesize βG mRNA with reduced translation capacity. The source of this difference must be genetically linked to the βG gene since the effect persists in the C57BL/6J.H congenic strain.

DISCUSSION

Identification of a rat cDNA sequence for βG, pRP1183, was based on its ability to selectively hybridize an RNA sequence capable of directing the in vitro synthesis of a polypeptide having the molecular weight and antigenic properties of authentic rat βG. Confirming evidence for this identification was provided by the fact that the cloned rat sequence cross hybridized with a mouse RNA having distinctive properties corresponding to those expected for βG mRNA.

Cloned mouse cDNA sequences were originally detected by hybridization with the previously isolated rat βG clone. Several criteria confirm the identity of the mouse cDNA clones. (1) The mouse clones hybridized to a mouse kidney RNA of 2.6 kb, the size expected for βG. (2) This RNA was strongly induced testosterone and induction followed the unusually slow kinetics previously demonstrated for βG protein synthesis and mRNA activity (Paigen et al., 1979; Watson et al., 1981). (3) Induction of this RNA was pituitary dependent, a property not seen for any of several other androgen-inducible kidney sequences that have been tested (Berger et al.,

TABLE I

Regulation of β-glucuronidase induction in kidney[a]

Mouse strain	[*Gus*] haplotype	Enzyme activity[b] (μ/g tissue)	Relative rate of enzyme synthesis[c] ($\times 10^3$)	βG mRNA[d] (μg/g total RNA)
A/J	A	303	1.93	18
C57BL/6J	B	179	1.17	7.5
C3H/HeJ	H	165	0.57	5.7
C57BL/6J.A	A	276	2.05	15.2
C57BL/6J.H	H	106	0.81	7.2

[a] Measurements of enzyme activity, rate of synthesis and mRNA concentration were done on the same animals using three pools of three animals each for each strain. One kidney from each animal was used for protein determinations, the other for RNA. All animals were induced with testosterone for 30 days.

[b] βG activity was determined by a fluorometric procedure using 4-methylumbelliferyl-β-glucuronide as substrate (Owerbach and Lusis, 1976). 1 u is defined as the production of 1 μmol 4-methylumbelliferone/h at 37°C; g tissue is wet weight.

[c] Relative rates of synthesis represent the fraction of total protein synthesis that was βG synthesis, and were determined by pulse-labeling experiments as described by Pfister et al. (1984) except that the concanavalin A step was omitted.

[d] βG mRNA concentrations were determined by hybridizing nick-translated [^{32}P]pGA-1 to 2-μg aliquots of kidney total RNA immobilized on nitrocellulose (Thomas, 1980). See MATERIALS AND METHODS, section **g**, for hybridization conditions. To determine relative concentrations of βG mRNA cpm hybridized were compared to a standard curve similar to that described in Fig. 5. Absolute concentrations were calculated based on a mass fraction of 1.8×10^{-5} for the RNA used as a standard (see RESULTS, section **f**).

1981; 1984). (4) The extent of induction of the hybridizing RNA was genetically determined by a locus, presumably *Gus-r*, closely linked to the [*Gus*] complex. (5) Using Southern blots of restricted genomic DNA probed with labeled pGA-1, polymorphisms for restriction fragment sizes were found between mice having the [*Gus*]^A and [*Gus*]^B haplotypes. Because the polymorphisms were carried by congenic strains of mice, the DNA sequences involved must map to the vicinity of the [*Gus*] complex.

The longest βG clone isolated, pGA-1, has been shown to have sequences in common with a mouse cDNA clone for βG constructed and identified by Palmer et al. (1983). The overlapping nature of these two independently derived clones was demonstrated by a similarity in restriction maps and by cross-hybridization (R. Ganschow, personal communication). A third independently derived clone also has a similar restriction map (Catterall and Leary, 1983).

In the course of these experiments a 1.0-kb fragment of cDNA from pGA-1 was subcloned into a vector carrying an SP6 RNA polymerase promoter. This allowed us to synthesize high-specific-activity, single-stranded RNA probes in vitro. These probes were specific for βG DNA and RNA sequences, and provided a means to quantitate βG mRNA concentrations even at very low levels. Using these probes the time course of induction was studied in detail. The pronounced lag after the administration of testosterone is much longer for βG mRNA than for several other androgen-inducible kidney RNAs (Berger et al., 1981; 1984). Throughout the lag and the subsequent induction, βG mRNA concentration followed a time course very similar to that previously determined by βG protein synthesis and mRNA activity (Watson et al., 1981). This indicates that the mechanism for induction does not involve the activation of pre-existing RNA, but rather the de novo accumulation of βG mRNA sequences. The 120-fold induction observed was similar to the extent of mRNA induction reported by Palmer et al. (1983), more than that reported by Catterall and Leary (1983), and somewhat less than the extent of induction as measured by rate of βG synthesis (Watson et al., 1981). Whereas the half-time for induction was about eight days, the half-life of βG RNA following the removal of testosterone from induced animals was about one day. Again, the changes in mRNA concentration as measured by hybridization were similar to changes in mRNA activity and rate of

protein synthesis measured previously (Watson et al., 1981).

Our results also bear on the nature of regulation by the *Gus-r* locus of βG induction by androgens. Present measurements of differences in hybridizable βG sequences in induced [*Gus*]^{A} and [*Gus*]^{B} mice agree with previous estimates of differences in in vivo rates of enzyme synthesis and in mRNA activity levels measured by translation in frog oocytes (Paigen et al., 1979; Watson et al., 1981). They are also in agreement with the results of Palmer et al. (1983). Differences in rates of βG synthesis between [*Gus*]^{B} and [*Gus*]^{H} mice, however, are not reflected in βG mRNA concentrations. Thus, whereas the difference between induced [*Gus*]^{A} and [*Gus*]^{B} mice reflects a quantitative difference in βG mRNA, the difference between [*Gus*]^{B} and [*Gus*]^{H} apparently reflects a qualitative difference. Presumably, βG mRNA from [*Gus*]^{H} mice is translated with a reduced efficiency. The determinants for both quantitative and qualitative differences in βG mRNA appear to be linked to the [*Gus*] gene complex on chromosome 5 because the various phenotypes are expressed in congenic strains of mice in which the original [*Gus*] complex has been replaced by the complex from strains carrying other haplotypes.

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