Two Genetic Elements Regulate Murine β-Glucuronidase Synthesis Following Transcript Accumulation

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ABSTRACT

Mutant alleles of two genetic regulatory elements, which underlie a three- to sixfold reduction in β-glucuronidase (GUS) activity levels, distinguish mice of the H haplotype from those of the other two common GUS haplotypes, A and B. Both elements are tightly linked to the GUS structural gene over which they exert control. One (*Gus-u*) exerts a *cis*-active effect upon GUS activity levels in all tissues at all times while the other (*Gus-t*) regulates GUS activity in *trans* after the 12th postnatal day in certain tissues. While previous studies show that differences in the rate of GUS synthesis account for the combined effects of these two elements in liver of adult mice, we demonstrate the separate effects of each on GUS synthesis at times during early postnatal development when their individual expressions can be distinguished. Assessments of the relative levels of S1 nuclease protection of a radiolabeled GUS antisense RNA probe after hybridization with total liver RNA preparations from adult mice of A and H haplotypes reveal no differences. These results argue that *Gus-u* and *Gus-t* exert their control of GUS expression subsequent to the accumulation of processed GUS transcripts.

GENETIC variations in β-glucuronidase (GUS) expression among inbred strains of laboratory mice provide a useful model system for the study of mammalian gene regulation. Natural variants of the hormonal, developmental and systemic regulation of GUS are associated with specific alleles of the GUS structural gene, *Gus-s*, and, together, define several haplotypes of the GUS gene complex, [*Gus*] [for a review of the genetics of murine GUS, see PAIGEN (1979); for definitions of common GUS haplotypes, see Table 1].

A well-characterized example of this variation is found in adult mice of the $[Gus]^h$ haplotype which are distinguished from mice of the other two common haplotypes, $[Gus]^a$ and $[Gus]^b$, by lower tissue levels of GUS activity (LAW, MORROW and GREENSPAN 1952; PAIGEN 1961a). This reduced level of GUS activity reflects changes in both a systemic and a developmental component of GUS expression (PAIGEN 1961a; LUSIS *et al.* 1983; WUDL and CHAPMAN 1976). Prior to the 12th postnatal day, all tissues of $[Gus]^h$ mice exhibit a three-fold lower level of GUS activity than those of $[Gus]^a$ and $[Gus]^b$ mice. Thereafter, GUS activity levels are reduced an additional three- to fourfold in liver, such that in adult $[Gus]^h$ mice the total reduction is six- to sevenfold (PAIGEN 1961a; LUSIS *et al.*, 1983; PFISTER *et al.* 1985).

Each of the two components of the reduced tissue levels of GUS activity in $[Gus]^h$ mice is controlled by a separate genetic element tightly linked to the GUS structural gene, *Gus-s* (PAIGEN 1961b; LUSIS *et al.* 1983). A *cis*-active systemic regulator, designated *Gus-u*, uniformly regulates GUS levels in all tissues throughout development, while a *trans*-acting developmental regulator, designated *Gus-t*, controls the postnatal accumulation of GUS activity in certain tissues at characteristic stages in development. To our knowledge, *Gus-t* is the only example of a *trans*-active cellular element which is tightly linked to the gene over which it exerts control. Alleles of *Gus-u* and *Gus-t* serve to partially define the common GUS haplotypes. Mice of the $[Gus]^h$ haplotype, which exhibit the reduced tissue levels of GUS activity, are $Gus\text{-}u^{h/h}$ and $Gus\text{-}t^{h/h}$ while mice of the $[Gus]^a$ and $[Gus]^b$ haplotypes are $Gus\text{-}u^{b/b}$ and $Gus\text{-}t^{a/a}$.

Previous studies demonstrated that the reduced levels of liver GUS in adult mice of the $[Gus]^h$ haplotype are directly related to reduced rates of GUS synthesis (GANSCHOW 1975; LUSIS *et al.* 1983). In the study reported here, analyses of GUS synthetic rates during the developmental period when the effects of *Gus-u* and *Gus-t* can be separated reveals that each element exerts its effect by altering the rate of GUS synthesis. Our results further demonstrate that the genetically altered synthetic rates are not the result of changes in either the transcription of *Gus-s* or the processing of GUS transcripts since adult levels of processed GUS transcripts are indistinguishable between $[Gus]^h$ and $[Gus]^a$ mice.

MATERIALS AND METHODS

Source of animals: Mice of the $[Gus]^a$ haplotype (A/J), $[Gus]^h$ haplotype (C3H/HeJ) and $[Gus]^b$ haplotype (C57Bl/6J) were purchased from The Jackson Laboratories (Bar

TABLE 1

Allelic definitions of the three common β-glucuronidase haplotypes and associated strains utilized in the present study[a]

GUS Haplotype	Alleles of			Selected Inbred or Congenic Strain
	Gus-s	*Gus-u*	*Gus-t*	
[*Gus*]^a	*a*	*b*	*a*	A/J B6.A-[*Gus*]^a
[*Gus*]^b	*b*	*b*	*a*	C57BL/6J
[*Gus*]^h	*h*	*h*	*h*	C3H/HeJ B6.C3H-[*Gus*]^h

[a] Information in this table is summarized from PAIGEN (1979) and from LUSIS *et al.* (1983).

Harbor, ME). The congenic inbred strains B6.A-[*Gus*]^a, B6.C3H-[*Gus*]^h, and (B6.A-[*Gus*]^a × B6.C3H-[*Gus*]^h)F_1 were obtained from Verne Chapman (Roswell Park Memorial Institute, Buffalo, New York). All adult mice were females of 60–90 days of age.

GUS activity assay: GUS activity was assayed by the procedure of MEISLER and PAIGEN (1972) using *p*-nitrophenyl-β-D-glucuronide (Cyclo Chemical Co.) as substrate. One unit of enzyme activity is defined as that amount of enzyme which releases 1 μmol of *p*-nitrophenol per hour at 56°.

Relative rate of GUS synthesis: Rates of GUS synthesis were determined according to the method of SMITH and GANSCHOW (1978) which measures the radioactivity present in immunoprecipitates of liver GUS at 2 hr after intraperitoneal administration of 20–200 μCi of L-[3,4-^{3}H]leucine (specific radioactivity 30–60 Ci/mmol; Amersham/Searle) as a fraction of the radioactivity incorporated into total trichloroacetic acid-insoluble material. Administered amounts of radioactivity varied as a function of the age of the mice. Mice were sacrificed in groups of three to 15 mice, depending upon age, and partially purified extracts were prepared from pooled livers of each group according to the method of SMITH and GANSCHOW (1978). Before purification, a portion of homogenate was reserved for assay of enzyme activity (see above) and for estimation of radioactivity incorporated into total trichloroacetic acid-insoluble material. Partially purified extracts contained greater than 90% of the original GUS activity.

A quantity of monospecific goat anti-GUS antibody, prepared and characterized as described by SMITH and GANSCHOW (1978), was added to the final tissue extract in amounts sufficient to precipitate all of the GUS activity. The resulting immunoprecipitates were solubilized, the components separated electrophoretically according to the method of WEBER and OSBORN (1969), and the GUS radioactivity in the gel determined by liquid scintillation spectrometry of 2 mm gel slices as described by SMITH and GANSCHOW (1978).

RNA preparation: Total RNA was prepared by homogenization of fresh mouse liver or kidney in guanidinium isothiocyanate followed by centrifugation through a cesium chloride cushion (CHIRGWIN *et al.* 1979). The RNA pellet was suspended in diethyl pyrocarbonate-treated water, and the RNA concentration determined by optical density measurements at 260 nm. RNA was stored at −70°.

Production of complementary RNA probes (cRNA): A 1.5-kb GUS cDNA (PALMER *et al.* 1983), representing *Gus-s* coding sequence from exon 4 through a portion of exon 12 (GALLAGHER *et al.* 1988; D'AMORE *et al.* 1988), was subcloned into a Bluescript vector (Stratagene) between the *Pst*I and *Hin*dIII sites. The insert was oriented such that transcription from the T7 RNA polymerase promoter yielded a ^{32}P-UTP labeled transcript complementary to the corresponding region of cellular GUS mRNA. Separation of the antisense RNA probe (cRNA) from the template was accomplished by agarose gel electrophoresis. The probe was purified from the gel and the specific activity determined. A 1.1-kb γ-actin cDNA (GUNNING *et al.* 1983) was subcloned in a similar manner at the *Pst*I and *Xba*I sites of Bluescript to generate a γ-actin cRNA for use as an internal control.

Hybridization, S1 nuclease treatment and hybrid analysis: The procedure by QUARLESS and HEINRICH (1986) was modified as follows: 100 μg of total liver or kidney RNA was precipitated with ethanol and the RNA pellet resuspended in formamide hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA and 40 mM MOPS pH 7.0). Approximately 1–5 × 10^5 cpm of each cRNA probe was added, and the volume adjusted to 20 μl, onto which 50 μl of mineral oil was layered. The sample was heated at 85° for 15 min to denature the RNA and then incubated at 65° for 3 hr to permit probe/RNA hybridization. Hybridization was followed by treatment with 400 units S1 nuclease (Boehringer Mannheim) at 37° for 1 hr to degrade single-stranded nucleic acids. Following digestion, the reaction was extracted with phenol/chloroform (1:1 v/v) and ethanol precipitated.

Following precipitation, the pellet was resuspended in 30 μl of 10 mM Tris, 1 mM EDTA (pH 8.0). To assay for hybrid molecules, the samples were electrophoresed at 175 V on a 5% native polyacrylamide gel. The gel was dried and autoradiography performed at −70° with an intensifying screen.

RESULTS

The differing patterns of accumulation of liver GUS activity between mice of the [*Gus*]^h and [*Gus*]^b haplotypes and their F_1 progeny, shown in Fig. 1A, confirm the previous results of PAIGEN (1961a) and of LUSIS and co-workers (1983). The three-fold difference in liver activity levels shortly after birth represents the effect of the *cis*-active *Gus-u* element while the sharp decline beginning at approximately day 12 in [*Gus*]^h mice and culminating in an additional three-to-four-fold difference in adult activity levels represents the effect of the *trans*-active element, *Gus-t* (LUSIS *et al.* 1983).

While previous studies demonstrate that the difference in adult activity levels between the haplotypes is related to a reduction in the rates of GUS synthesis (GANSCHOW 1975; LUSIS *et al.* 1983), analyses of the mechanisms by which *Gus-u* and *Gus-t* exert their effects requires examination of GUS synthesis at times when the effects of these elements can be separated. Since the effects of *Gus-t* are not observed until 10–15 days postpartum whereas *Gus-u* is expressed at all times in all tissues (LUSIS *et al.* 1983), we compared GUS activity levels (Figure 1A) with GUS synthetic rates (Figure 1B) in liver during postnatal development for [*Gus*]^b and [*Gus*]^h mice and for their F_1 progeny. It can be seen that the relative pattern of GUS synthetic rates is similar to that of GUS activity levels for each category of mice during postnatal development. Thus, both *Gus-u* and *Gus-t* exert con-

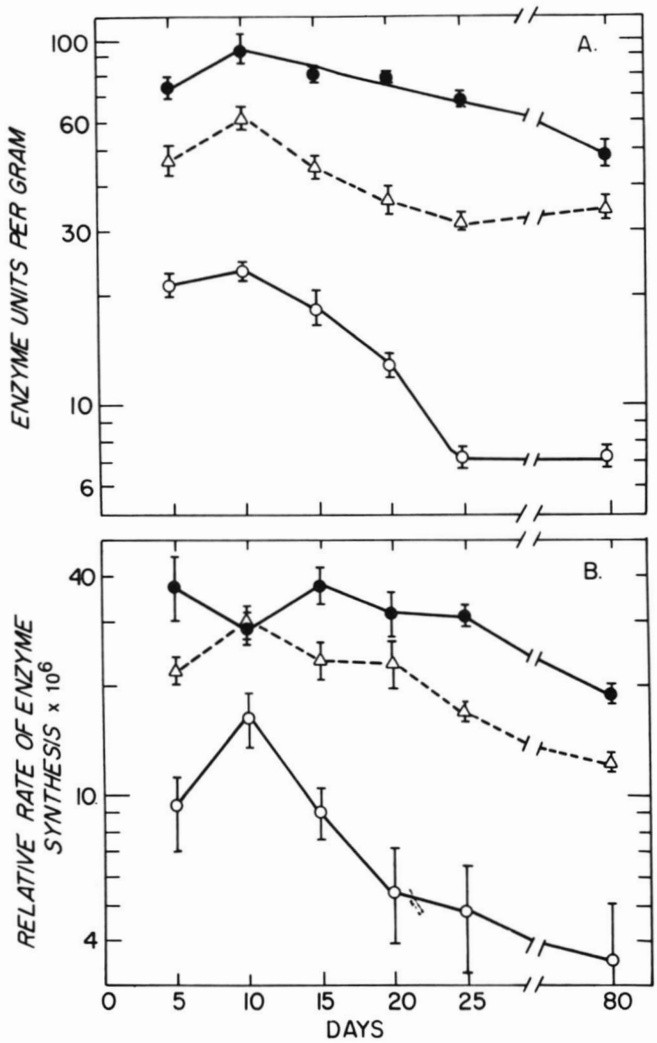

FIGURE 1.—Comparison of activity levels and relative rates of synthesis of liver GUS among mice of the $[Gus]^b$ and $[Gus]^h$ haplotypes and their F_1 progeny during postnatal development. Activity levels and relative rates of synthesis were determined as described in MATERIALS AND METHODS and are plotted as the mean values of at least three pools of livers ± SEM. Closed circles represent values for mice of the $[Gus]^b$ haplotype (C57BL/6J strain), open circles those for $[Gus]^h$ haplotype (C3H/HeJ strain) mice, and triangles those for C57BL/6J-$[Gus]^b$ × C3H/HeJ-$[Gus]^h$ F_1 mice.

trol over the expression of *Gus-s* by regulating the rates of GUS synthesis.

To determine whether *Gus-u* and *Gus-t* regulate GUS synthesis by altering levels of GUS transcripts, we assessed the extent of S1 nuclease protection of a GUS antisense RNA by liver RNA from adult mice. Previous attempts to assess the constitutive levels of GUS mRNA have been difficult using standard RNA blot hybridization procedures due to the low abundance of such transcripts. However, highly radioactive, GUS RNA probes and their use in conjunction with S1 nuclease protection analyses permit us to measure less than ten pg of GUS mRNA in 100 μg of total RNA, based upon previous estimates of the abundance of GUS transcripts in the liver (PFISTER *et al.* 1985).

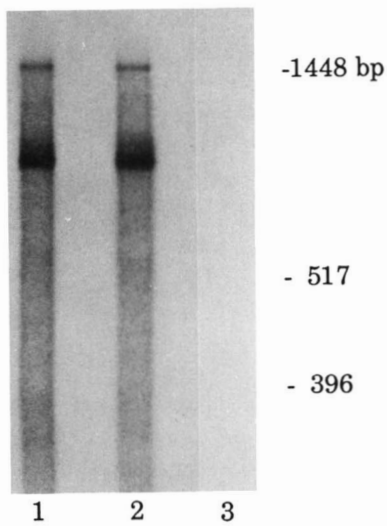

FIGURE 2.—Comparison of levels of GUS liver transcripts between strains of inbred mice representing the $[Gus]^a$ and $[Gus]^h$ haplotypes. RNA samples were analyzed as described in MATERIALS AND METHODS for the level of their ability to protect the 1.5-kb GUS and 1.1-kb γ-actin antisense RNAs. Haplotypes and strains of origin of liver RNAs are: lane 1, $[Gus]^a$, A/J; lane 2, $[Gus]^h$, C3H/HeJ. Lane 3 represents total yeast RNA.

Total liver RNA preparations isolated from mice with high (*Gus-u*$^{b/b}$, -*t*$^{a/a}$) and low (*Gus-u*$^{h/h}$, -*t*$^{h/h}$) adult levels of GUS synthesis were hybridized with a 1.5-kb radiolabeled GUS antisense RNA, digested with S1 nuclease and electrophoresed in polyacrylamide gels as described in MATERIALS AND METHODS. Autoradiographic comparisons of the gel, shown in Figure 2, reveal relatively equivalent protection of the 1.5-kb GUS riboprobe by the two RNA preparations under conditions where yeast total RNA offers no protection. That the relative integrity and quantities of the liver RNA preparations are equivalent is demonstrated by protection of a radiolabeled 1.1-kb mouse γ-actin riboprobe included in the same reaction mixtures. Densitometric analyses reveal that the relative levels of protection of the 1.5-kb GUS and the 1.1-kb γ-actin antisense RNA are equivalent for liver RNAs from mice of each of the GUS haplotypes represented in Figure 2. Since the protected sequence observed in these experiments represents 56% of the GUS mRNA sequence and traverses splice junctions of *Gus-s* between exons 4 and 12 (GALLAGHER *et al.* 1988, D'AMORE *et al.* 1988), the GUS antisense cRNA probe therefore detects processed GUS transcripts but not unprocessed or partially processed transcripts.

In a separate experiment, we compared congenic strains representing the two GUS haplotypes on C57BL/6 backgrounds, B6.A-$[Gus]^a$ and B6.C3H-$[Gus]^h$, and their heterozygotes, (B6.A-$[Gus]^a$ × B6.C3H-$[Gus]^h$)F_1. Results of such comparisons, shown in Figure 3, reveal that the relative level of protection of GUS and γ-actin antisense RNA by total

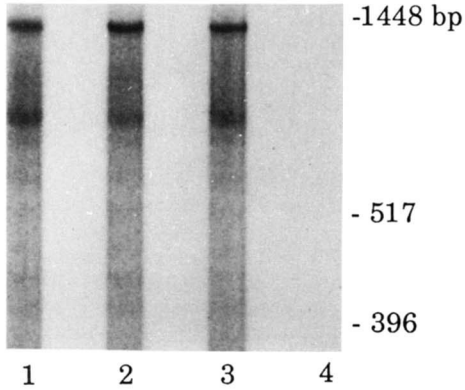

FIGURE 3.—Comparison of levels of GUS liver transcripts among congenic strains of mice representing the $[Gus]^a$ and $[Gus]^h$ haplotypes, and their F_1 progeny. RNA samples were analyzed as described in MATERIALS AND METHODS for the levels of their ability to protect the 1.5-kb GUS and 1.1-kb γ-actin antisense RNAs. Congenic strains of origin (with haplotype designation) of liver RNAs are: lane 1, B6.C3H-$[Gus]^h$; lane 2, (B6.C3H-$[Gus]^h$ × B6.A-$[Gus]^a$) F_1; lane 3, B6.A-$[Gus]^a$. Lane 4 represents total *E. coli* RNA.

liver RNA is equivalent between the $[Gus]^a$ and the $[Gus]^h$ congenic strains.

It should be pointed out that the specific activities of the GUS and γ-actin antisense RNAs differ between experiments as well as between one another in the experiments represented in Figures 2 and 3. In each experiment, the ratio of intensities of the protected GUS fragment is equivalent to that of the protected γ-actin fragment for the two haplotypes. Differences in the relative intensity levels of GUS and γ-actin from experiment to experiment are an artifact of differences in specific radioactivity of the two riboprobes and are not a measure of the relative levels of the two transcripts.

Two lines of evidence demonstrate that the nuclease protection assay is capable of discriminating differences in levels of GUS mRNA. First, increasing amounts of total liver RNA added to the reaction mixture give proportional increases in the amount of protected riboprobe (Figure 4). Second, when GUS activity is stimulated by androgen to high levels in kidneys of female $[Gus]^a$ mice, we observe a dramatic increase in the levels of riboprobe protection by RNA from kidneys of treated animals when compared to that of untreated mice (Figure 5). The extent of this difference is consistent with that of previous studies on the androgen responsiveness of kidney GUS mRNA in $[Gus]^a$ mice (PALMER *et al.* 1983; WATSON *et al.* 1981).

DISCUSSION

Our results demonstrate that the murine β-glucuronidase (GUS) regulatory elements, *Gus-u* and *Gus-t*, while exerting control over the rate of synthesis of GUS, do not influence the tissue levels of GUS mRNA. Since the rates of synthesis of liver GUS were measured by comparing the fraction of total radioactive protein present in immunoprecipitable GUS after a short in vivo pulse of radiolabeled amino acids, the effects of *Gus-u* and *Gus-t* on GUS synthetic rates must occur subsequent to the processing of GUS transcripts, but prior to the recognition and binding of GUS by specific antibodies.

Although not a common mode of regulation of eukaryote gene expression, translational mechanisms have been identified (MATHEWS 1986; LODISH 1976). When operative, translational regulation usually occurs at initiation (JAGUS, ANDERSON and SAFER 1981), with only rare examples of mechanisms involving elongation (THOMAS and MATHEWS 1984) or termination (YOSHIYUKI *et al.* 1985) of mRNA. Evaluation of the parameters responsible for the differential expression of GUS between murine tissues suggest that such differences arise primarily from alterations in translational yield which is defined as the number of mature enzyme molecules formed per minute per mRNA molecule (BRACEY and PAIGEN 1987). Variations in translational yield can be due to alterations in either translational efficiency, which is generally a consequence of initiation, or an event which promotes the maturation of GUS polypeptides to a point where they can be recognized by GUS antibodies.

A strong inference of our results is that the *Gus-u* regulatory element resides within exon sequences of *Gus-s* and specifies a recognition site either within the GUS mRNA or the GUS polypeptide. If mutant *Gus-u* and *Gus-t* alleles in H haplotype mice alter the rates of GUS synthesis without altering the level of GUS transcripts, then neither element can exert its effect through a transcriptional mechanism. Thus, the *cis*-active *Gus-u* element, which only regulates the expression of that *Gus-s* allele on the same DNA molecule, must encode a recognition site in the processed GUS transcript or in GUS itself. If located within the mRNA, then this recognition site could vary between the GUS haplotypes such that transcripts derived from the wild-type $Gus\text{-}u^b$ allele are translated more efficiently relative to those from the $Gus\text{-}u^h$ allele. Differences between transcripts involving primary sequences and/or secondary structure around the initiation codon are known to alter the rate of attachment to ribosomes and/or other stages of translation initiation (LODISH and JACOBSEN 1972; LEE, GUERTIN and SONENBERG 1983).

The *trans*-active *Gus-t* element, which exerts tissue-specific control over GUS expression shortly after birth, must by definition exert its effect through a gene product (HERRUP and MULLEN 1977; MEREDITH and GANSCHOW 1978; LUSIS *et al.* 1983). MEREDITH and GANSCHOW (1978) proposed that this product is the GUS polypeptide itself (or its mRNA) and that the temporal control of liver GUS expression by *Gus-*

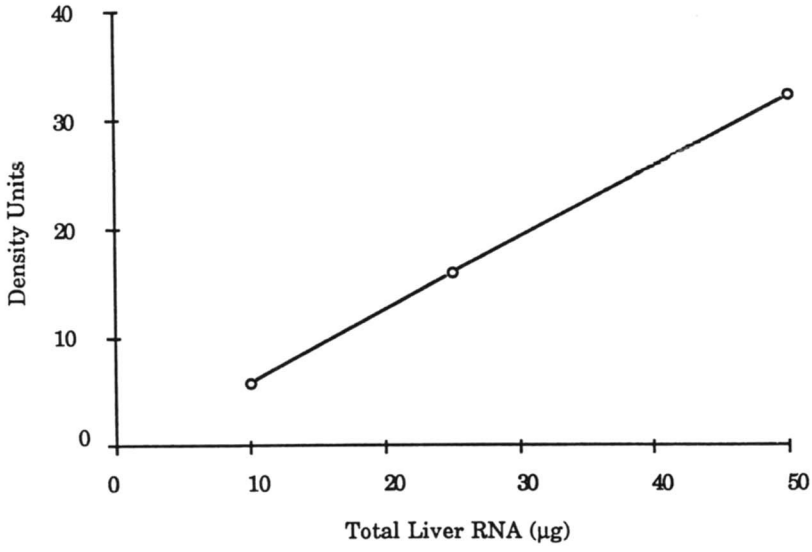

FIGURE 4.—Nuclease protection of the GUS antisense RNA by increasing amounts of total liver RNA. Serial dilutions of total liver RNA from mice of the A/J strain were analyzed for the levels of their ability to protect the 1.5-kb GUS antisense RNA as described in MATERIALS AND METHODS. The relative intensities of the autoradiographic bands representing the protected fragment were measured by scanning densitometry and plotted as arbitrary density units *vs.* the relative level of input RNA for each dilution.

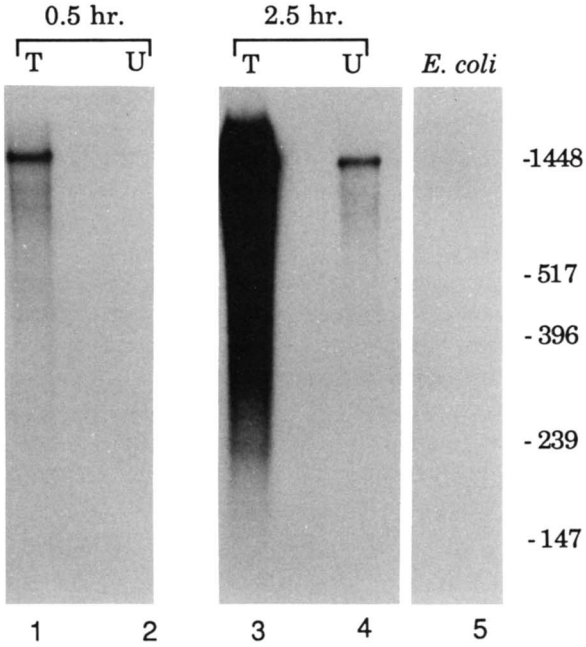

FIGURE 5.—Comparison of the levels of nuclease protection of the GUS antisense RNA by total RNA preparations from untreated and androgen-treated female mice of the [*Gus*]^a haplotype. Ten adult female mice of the A/J strain were divided into two groups. A 75-mg pellet of testosterone was implanted subcutaneously into individuals of one group. Twenty-one days later all animals were sacrificed and kidneys of each treatment group pooled. Total RNAs from each pool were prepared and analyzed for the levels of their ability to protect the 1.5-kb GUS antisense RNA as described in MATERIALS AND METHODS. Autoradiographic films were exposed for either 0.5 or 2.5 hr as indicated. The source of kidney RNA is: lanes 1 and 3, testosterone-treated mice; lanes 2 and 4, untreated mice. Lane 5 represents total RNA from *E. coli*.

t represents an autoregulatory phenomenon. Autoregulatory mechanisms have been proposed to explain the control of other eukaryotic genes. Transient DNA transfection assays of chimeric genes have identified a cytoplasmic autoregulatory mechanism that permits the level of β-tubulin transcripts to be modulated by the apparent concentration of unpolymerized tubulin subunits in mouse L cells (GAY *et al.* 1987). Studies on the regulation of *suppressor-of-white-apricot* expression in Drosophila reveal a post-transcriptional mechanism which also appears to be autoregulatory (CHOU, ZACHAR and BINGHAM 1987; ZACHAR, CHOU and BINGHAM 1987).

It is therefore possible that *Gus-u* and *Gus-t* both reside within coding regions of the GUS structural gene, each serving to specify recognition sites within either GUS mRNA or its polypeptide. Furthermore, the H haplotype allele of *Gus-u* could be distinguished from that of other GUS haplotypes by the same mutation which distinguishes the H haplotype allele of *Gus-t*. If correct, then regulatory models which describe the synthesis of GUS in mice of the H haplotype would need to account for a single mutation with both *cis* and *trans* regulatory manifestations. It is conceivable that in mice of the H haplotype, recognition sites specified by *Gus-u* and *Gus-t* are encoded within exon sequences of *Gus-s* which surround or lie within such a mutation. As a result of changes in each recognition site imparted by the single mutation, the efficiency of translation and/or an event immediately distal to translation could be altered and thus account for the observed differences in the rates of GUS synthesis between the H haplotype and other GUS haplotypes.

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