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Androgen Responsiveness of the Murine β-Glucuronidase Gene Is Associated with Nuclease Hypersensitivity, Protein Binding, and Haplotype-Specific Sequence Diversity within Intron 9

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The tissue specificity and genetic variability of the murine β-glucuronidase (GUS) response to androgen provide useful markers for identifying elements which underlie this responsiveness. While GUS is expressed constitutively in all examined cell types, kidney epithelial cells uniquely exhibit a manyfold yet slow rise in GUS mRNA and enzyme levels when stimulated by androgens. Three major phenotypes of this androgen response have been described among inbred strains of mice: (i) a strong response in strains of the *Gus*^{a} haplotype, (ii) a reduced response in strains of the *Gus*^{b} and *Gus*^{h} haplotypes, and (iii) no response, as observed in *Gus*^{or} mice. These response variants define a *cis*-active element(s) which is tightly linked to the GUS structural gene. Nuclease hypersensitivity scans of kidney chromatin within and surrounding the structural gene revealed an androgen-inducible hypersensitive site in intron 9 of the gene in *Gus*^{a} but not in *Gus*^{or} mice. When a radiolabeled fragment of *Gus*^{a} DNA containing this hypersensitive site was incubated with kidney nuclear extracts and then subjected to gel electrophoresis, two shifted bands were observed whose levels were dramatically higher in extracts of androgen-treated than in those of untreated *Gus*^{a} mice. The shifted bands reflect binding of a kidney-specific factor(s) to a 57-bp region of complex dyad symmetry in *Gus*^{a} and *Gus*^{or} mice which is partially deleted in *Gus*^{b} and *Gus*^{h} mice. This binding site is located approximately 130 bp downstream of a glucocorticoid response element sequence motif which is totally deleted in [*Gus*]^{or} mice. Taken together, our results suggest that the androgen responsiveness of GUS in murine kidney epithelial cells is controlled by elements within the proximal end of intron 9 of the GUS structural gene.

The effects of androgens on mammalian tissues are varied and numerous. Many examples of specific responses of genes to androgen treatment have been described (reviewed in reference 4). Cloning and characterization of the human and rat androgen receptor genes provide support for the view that the primary effects of androgens are generated at the level of gene transcription and are mediated by the interaction of a steroid-receptor complex and a *cis*-acting DNA sequence in the vicinity of the responding gene (11, 29). Like other steroid receptors, the androgen receptor contains a highly conserved DNA binding domain, a carboxyl-terminal steroid binding domain, and an amino-terminal variable domain. Sequences similar to the consensus glucocorticoid response element (GRE) and progesterone response element have been identified within and upstream of several androgen-responsive genes (14, 25, 35).

Mouse kidney, one of the more androgen-responsive tissues, offers several features which facilitate the study of androgenic effects (reviewed in references 2 and 4). In contrast to tissues of the reproductive tract, testosterone is not converted to 5α-dihydrotestosterone by mouse kidney, thus simplifying the interpretation of androgen effects. Moreover, androgens do not stimulate DNA synthesis in the kidney, permitting the study of cellular hypertrophy free of the effects of hyperplasia. In addition, a number of kidney enzymes are induced to moderate levels of activity by androgen. This contrasts with some other cell types in which steroid treatment induces large amounts of a few specific proteins.

We are analyzing the responsiveness of the murine β-glucuronidase (GUS) gene to androgens because the tissue specificity and genetic variability of this steroid response system provide useful markers for identifying factors and elements which mediate the response. While GUS is expressed constitutively in all examined cell types, kidney epithelial cells uniquely exhibit a manyfold yet slow rise in GUS mRNA and enzyme levels when stimulated by androgens (reviewed in reference 33). Three major phenotypes of this androgen response have been described among inbred strains of mice: (i) a strong response after a 1-day lag in strains of the *Gus*^{a} haplotype, (ii) a reduced response associated with a 2-day lag in strains of the *Gus*^{b} and *Gus*^{h} haplotypes, and (iii) no response, as observed in *Gus*^{or} mice (30, 38, 40). These response variants define a *cis*-active element(s) which is required for the androgen responsiveness of GUS but which has no detectable effect upon basal levels of GUS enzyme or its mRNA.

The androgen response of GUS requires the androgen receptor. *tfm* mutant mice, which lack functional receptors, exhibit no GUS response to androgen (16, 17). GUS responds in a highly specific manner to androgenic steroids (20) but does not respond to either glucocorticoid or progesterone (21). On the other hand, the GUS response to androgen is antagonized by estrogen (21). While the major mechanism of the GUS response appears to be at the level of

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transcription, as suggested by studies of the rates of GUS mRNA synthesis, androgen may also serve to stabilize GUS mRNA to some degree (41).

Growth hormone plays an important role in the androgen response of GUS. In hypophysectomized mice and in the dwarf (*dw*) mouse, which lacks anterior pituitary function, the extent of the GUS response is reduced to approximately 25% of normal (37). However, no effect is observed on basal levels of GUS. That growth hormone deficiency underlies this reduced response to androgen is suggested by loss of the GUS response in the little (*lit*) mouse, which is deficient in growth hormone and prolactin, and by the restoration of this loss upon administration of growth hormone but not prolactin. Growth hormone is not a common requirement for androgen-responsive genes in kidney, since inductions of a number of other androgen-responsive gene products are unaffected by growth hormone (33).

To identify and characterize those elements and factors which mediate the androgen response of GUS, we are analyzing DNA sequences within and surrounding *Gus-s* among the various response haplotypes. In the initial studies reported here, we present results of nuclease hypersensitivity, gel binding, and DNA sequence analyses.

MATERIALS AND METHODS

DNase I hypersensitivity assessments. To prepare nuclei, approximately 2 g of tissues from female mice were homogenized (Dounce) in 12 ml of buffer A (1 M sucrose, 1 mM sodium *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.95], 4 mM $MgCl_2$, 0.1 mM EGTA, 2 mM β-mercaptoethanol) and filtered through 290- and 110-μm plastic mesh (Spectrum); nuclear pellets isolated according to the method of Hewish and Burgoyne (26). Except for the zero-time incubations, aliquots (0.4 ml) containing approximately 2×10^7 nuclei were treated with 0.014 U of DNase I at 37°C. At the times indicated in Fig. 1, all reactions were stopped by the addition of 100 μl of 0.2 M EDTA–2.5% sodium dodecyl sulfate (SDS) and the DNA was purified. Twenty micrograms of each DNA preparation was digested with appropriate restriction enzymes, subjected to electrophoresis in a 0.7% agarose gel, and transferred to Zetabind. The transferred DNA was hybridized with an appropriate end-specific radioactive probe. The radiolabeling procedure was that of Feinberg and Vogelstein (19). Blots were incubated overnight at 42°C in 30 ml of prehybridization solution (6× SSC [1× is 0.15 M NaCl plus 0.015 M sodium citrate], 10× Denhardt's solution, 1% SDS, 100 μg of denatured salmon sperm DNA per ml) and then incubated for 24 to 48 h at 42°C in 20 ml of hybridization solution (6× SSC, 1% SDS, 100 μg of denatured salmon sperm DNA per ml, 50% formamide, 5% dextran sulfate). Blots were first rinsed at room temperature (RT) in 2× SSC–0.1% SDS and then washed for 15 min at RT in 2× SSC–0.1% SDS, for 15 min at RT in 0.1× SSC–0.1% SDS, and for 30 min at 65°C in 0.1× SSC–0.1% SDS. The blots were then autoradiographed for 3 to 7 days.

DNA sequencing. Five hundred nanograms of genomic DNA representing each GUS haplotype was used as a template for the polymerase chain reaction (PCR), using the primers shown in Fig. 5. To ensure that the ends of the PCR products were blunt, an aliquot of the PCR reaction mix was heated at 100°C for 10 min to inactivate *Taq* polymerase, the $MgCl_2$ concentration was adjusted to 5 mM, and 2.5 U of DNA polymerase I (Klenow fragment) was added; the extension was allowed to proceed for 20 min at RT. DNA polymerase I (Klenow fragment) was inactivated by heating at 100°C for 10 min. The PCR product was digested with *Bam*HI (Fig. 5, residue 7776), and the *Bam*HI 3′-end fragment was ligated into M13mp18 and sequenced by the quasi-end-labeling adaptation of the dideoxy-chain termination method (18).

Gel shift assays. Gel shift assays were carried out by using the Stratagene GelShift Kit as instructed by the manufacturer. Soluble kidney and liver nuclear extracts were prepared by a modification of the method of Hodo and Blatti (27). All procedures were performed at 4°C or on ice. Kidneys and livers were removed, cleaned of membranes, and weighed. Five milliliters of AB buffer (25 mM Tris [pH 7.9], 12 mM KCl, 5 mM $MgCl_2$, 7 mM β-mercaptoethanol, 10% glycerol, 5 μg of aprotinin [Sigma] per ml, 25 μg of phenylmethylsulfonyl fluoride [PMSF; Sigma] per ml) was added per g of tissue prior to homogenization. Aprotinin and PMSF were added just prior to use. Homogenization took place for 30 s at medium speed, using the SDT 182E generator of a Tissumizer (Tekmar Co., Cincinnati, Ohio). Extracts were then centrifuged at 13,000 × *g* for 10 min. The supernatant was transferred to a clean tube, and 4 μl of polyethyleneimine (a 10% neutralized solution; Aldrich) was added dropwise per ml of extract. The mixture was stirred for 30 min and then centrifuged (13,000 × *g*, 10 min). The pellet was then extracted with 2.5 ml of C buffer (50 mM Tris [pH 7.9], 25 mM KCl, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol) containing 200 mM NH_4SO_4, 5 μg of aprotinin per ml, and 25 μg of PMSF per ml. Extraction was performed by using a Dounce homogenizer, and the extracts were stirred for 30 min. After centrifugation (11,000 × *g*, 10 min), the supernatant was stored at −70°C in small aliquots. Protein concentration of the nuclear extracts was determined by using the Bio-Rad protein assay kit.

Extracts containing 5 μg of protein were tested for the presence of factors which bind to 1 ng (5×10^3 to 8×10^3 cpm) of a fragment of Gus^a or Gus^{or} DNA prepared by PCR in the presence of [α-^{32}P]dATP. These fragments, whose ends were defined by primers shown under the heavy arrows in Fig. 5, are located within the proximal end of intron 9 and include DNase I-hypersensitive (DH) site 7.9. The DNA fragment alone and after incubation with extract was subjected to electrophoresis in a 4% native polyacrylamide gel in 1× Tris-glycine buffer (50 mM Tris-HCl [pH 8.5], 0.38 M glycine, 2 mM EDTA) at 30 mA for 3 h at 4°C. Triton X-100 (0.1%) was included in the gel presented in Fig. 11. Gels were dried and autoradiographed overnight.

RESULTS

An androgen-inducible DH site in intron 9 of *Gus-s*. Since nuclease-hypersensitive sites are frequently associated with *cis*-acting regulatory elements and since relatively large regions of DNA can be quickly scanned for such sites, we initiated these studies by examining chromatin within and surrounding the GUS structural gene, *Gus-s*, for DNase I hypersensitivity. Of five DH sites identified within and surrounding *Gus-s* (29a), one was androgen responsive. It was observed in chromatin of liver and kidney cells from mice treated with androgen for 14 days but not in samples from untreated mice of the Gus^a haplotype. This androgen-inducible DH site (site 7.9), located within the proximal end of intron 9, was verified by examining an 8-kb *Kpn*I restriction fragment of Gus^a DNA in both directions, using end-specific, radiolabeled probes (Fig. 1). The same 8-kb *Kpn*I restriction fragment also contained a constitutive DH site

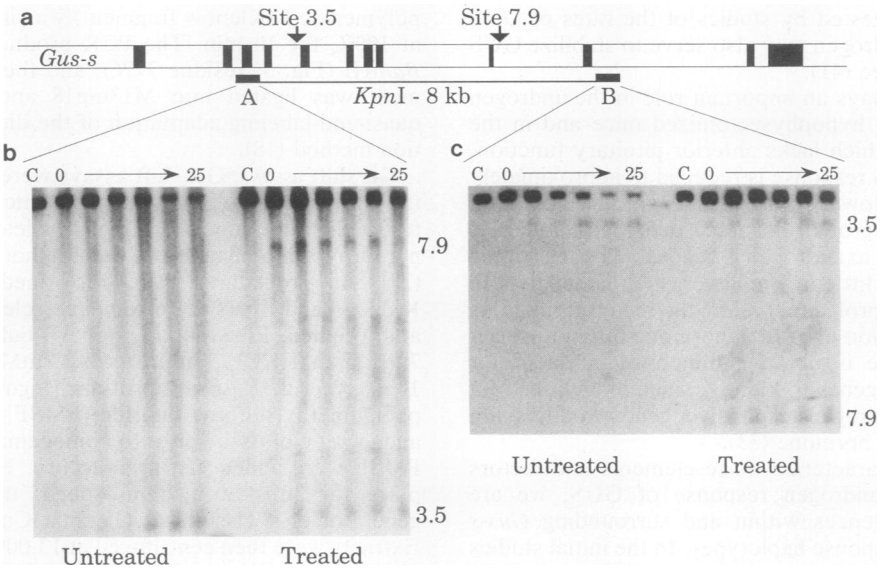

FIG. 1. DNase I hypersensitivity of kidney chromatin within an 8-kb *Kpn*I fragment of the A-haplotype allele of *Gus-s*. (a) Graphic depiction of the GUS structural gene (*Gus-s*). Filled areas represent each of the 12 exons of the 14-kb gene. (b) Kidney DNA from androgen-treated and untreated A/J mice hybridized with probe A (5′ probe). (c) The same blot stripped and hybridized with probe B (3′ probe). The strong band at the top of each lane is the 8-kb *Kpn*I parent fragment. Each treatment group contains seven lanes, the leftmost of which is spleen genomic DNA not subjected to DNase I treatment (indicated as lane C in each group). The remaining lanes represent increasing times of DNase I treatment in intervals of 5 min, indicated as 0 → 25 for each group. Designations of the two DH sites as DH site 3.5 and DH site 7.9, as indicated in panel a, are based on their positions in kilobases from the transcriptional start site of *Gus-s*.

(site 3.5) within intron 4 of *Gus-s* which was found in kidney chromatin of both treated and untreated mice, thus providing a positive control for chromatin of untreated mice. Liver chromatin of mice treated with androgen for 14 days also exhibited the androgen-inducible DH site 7.9 (Fig. 2), even though liver GUS does not respond to androgen.

Other hybridizing fragments were observed sporadically (Fig. 1 and 2). The corresponding fragments, however, were never observed when the same blot was probed in the other direction (Fig. 1), an indication that these do not represent additional inducible DH sites. Thus, the only inducible DH site consistently observed in kidney and liver chromatin of A haplotype mice is located in the proximal end of intron 9.

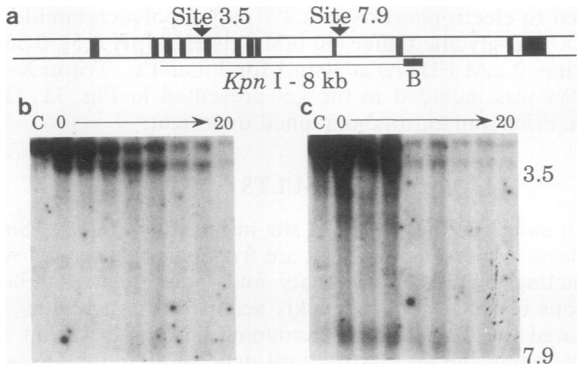

FIG. 2. DNase I hypersensitivity of liver chromatin within an 8-kb *Kpn*I fragment of the A-haplotype allele of *Gus-s*. (a) The GUS structural gene, depicted as in Fig. 1. (b and c) Liver DNA from untreated (b) androgen-treated (c) A/J mice hybridized with probe B. The strong band at the top of each lane is the 8-kb *Kpn*I parent fragment. Each treatment group contains nine lanes, the leftmost of which is spleen genomic DNA not treated with DNase I (indicated as lane C in each group). The remaining lanes represent increasing times of DNase I treatment in intervals of 2.5 min, indicated as 0 → 20 for each group. DH site 3.5 and 7.9 are labeled next to each group and are indicated in panel a.

The digestibility of the parent band in kidney chromatin of untreated mice appeared to progress more rapidly than that of treated mice, possibly leading to a more rapid rate of loss and nondetection of fragments representing DH site 7.9. Based on two other observations, however, we consider this possibility highly unlikely. First, similar levels of fragments representing the constitutive DH site 3.5 were observed between digests of treated and untreated kidney and liver chromatin (Fig. 1 and 2). Second, in contrast to the situation in kidney, digestibility of the parent band in liver chromatin of untreated mice appeared somewhat less rapid than that of treated mice. Nevertheless, no fragment representing DH site 7.9 was observed in digests from untreated mice (Fig. 2).

Since the androgen-inducible DH site 7.9 located within intron 9 of *Gus-s* was correlated with the androgen inducibility of kidney GUS in *Gus*^{a} mice, it was of interest to test whether the same site existed within the chromatin of mice of the nonresponding OR haplotype. Since *Gus*^{or} mice exhibit a polymorphism with respect to *Kpn*I, it was necessary to use a different restriction fragment to test for DH site 7.9 in *Gus*^{or} mice. As the parent fragment we chose a 7-kb *Hin*dIII fragment which extends from the middle of intron 8 through the middle of exon 12 (Fig. 3). The results of this test (Fig. 3) reveal the expected 1.2-kb band in *Gus*^{a} digests of kidney nuclei of androgen-treated mice as the marker for DH site 7.9. However, the same band was not revealed in digests from kidney or liver nuclei of either treated or untreated *Gus*^{or} mice.

Because the 7-kb *Hin*dIII fragment contains no constitutive DH site as was found in the *Kpn*I fragment examined in *Gus*^{a} mice, it was possible that the *Gus*^{or} chromatin was not

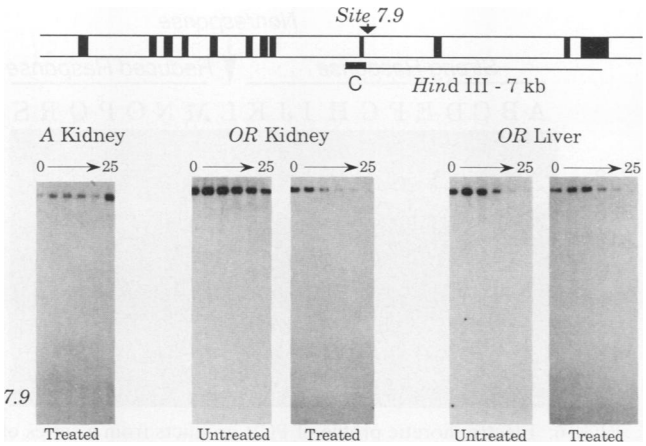

FIG. 3. DNase I hypersensitivity of kidney and liver chromatin within a 7-kb *Hin*dIII fragment. The GUS structural gene is depicted as in Fig. 1. The panels display kidney and liver DNA from androgen-treated and untreated OR haplotype mice and kidney DNA from treated mice of the A haplotype hybridized with probe C (1,050-bp *Hin*dIII-*Bal*I fragment). The band at the top of each lane is the 7-kb *Hin*dIII parent fragment. Each treatment group contains six lanes which represent increasing times of DNase I treatment in intervals of 5 min, indicated as 0 → 25 for each group. DH site 7.9 is indicated in the A haplotype kidney panel, and its position is shown within the diagram of *Gus-s*.

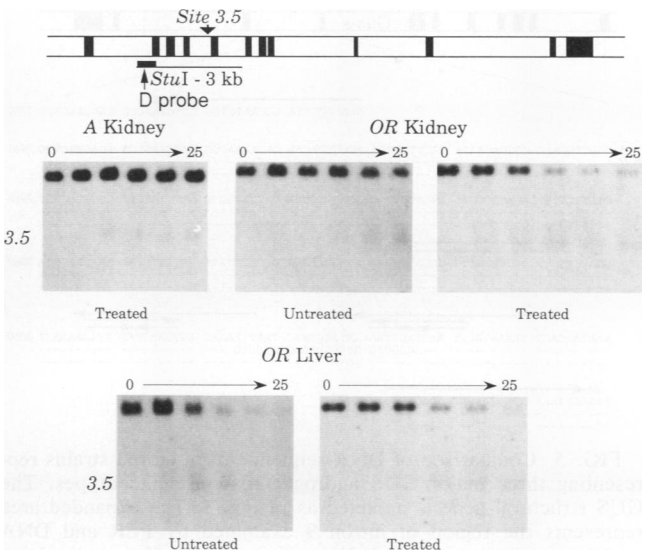

FIG. 4. DNase I hypersensitivity of kidney and liver chromatin within a 3-kb *Stu*I fragment of *Gus-s*. The GUS structural gene is depicted as in Fig. 1. The panels display kidney and liver DNA from androgen-treated and untreated OR haplotype mice and kidney DNA from treated mice of the A haplotype hybridized with probe D (895-bp *Stu*I-*Kpn*I fragment). The band at the top of each lane is the 3-kb *Stu*I parent fragment. Each treatment group contains six lanes which represent increasing times of DNase I treatment in intervals of 5 min, indicated as 0 → 25 for each group. DH site 3.5 is indicated in the A haplotype kidney panel, and its position is displayed within the diagram of *Gus-s*.

amenable to digestion and thus incapable of revealing the appropriate marker for DH site 7.9. On the basis of the following observations, we believe that this is not the case. First, the data of Fig. 3 reveal that the 7-kb parent band of Gus^{or} is readily digested. Second, the same Gus^{a} and Gus^{or} DNA preparations used in Fig. 3, when digested with *Stu*I, revealed the marker fragment for DH site 3.5 (Fig. 4).

Haplotypic differences in GUS response to androgen are associated with sequence alterations in the region of intron 9 containing DH site 7.9. Previous studies from our laboratory revealed an A+T-rich, 57-bp DNA sequence exhibiting complex dyad symmetry in Gus^{a} mice (13) which is located within the same region of intron 9 identified in these studies by nuclease hypersensitivity. If the targeted region represents a *cis*-active androgen response element, then haplotype-specific differences in the response of GUS should be reflected in DNA structural differences. To test this prediction, DNA sequence in the targeted region was determined for one or more representative inbred strains of each androgen response phenotype (Fig. 5).

Examination of DNA from Gus^{b} and Gus^{h} strains, which exhibit the reduced response to androgen, revealed a 22-bp deletion within the sequence of complex dyad symmetry observed for Gus^{a} mice as well as a transversion (A to T) at residue 7973. Nonresponding Gus^{or} mice exhibited a 28-bp deletion approximately 150 bp upstream from the region of dyad symmetry between positions 7832 and 7859, two single-base-pair deletions at residues 8051 and 8052, and three single-base-pair substitutions at residues 7820, 7960, and 8012.

Further examination of the data presented in Fig. 5 revealed a sequence in DNAs of the androgen-responsive Gus^{a} and Gus^{b} haplotypes (residues 7833 through 7847) which exhibited a strong similarity to the consensus GRE 5′-GGTACAnnnTGTTCT-3′ (3), and which was entirely deleted in Gus^{or} haplotype DNA.

If the DNA sequence differences within the targeted region observed among the androgen response phenotypes underlie the differences in response, then complete concordance should be observed between the sequence and response differences for inbred strains previously characterized with respect to response phenotype. To test this prediction, DNA in the targeted region was amplified by PCR (36) for 1 nonresponse, 7 reduced-response, and 11 strong-response strains and then analyzed by agarose gel electrophoresis (Fig. 6) and DNA sequencing (Fig. 5). Ten of the eleven strong-response strains contained a fragment whose size indicated an intact 57-bp complex repeat, while all of the reduced-response strains exhibited a fragment whose size correlated with the 22-bp deletion. The single-nucleotide variation at residue 7973 and the presence or absence of the 22-bp deletion were confirmed by sequencing in three strong-response and four reduced-response strains. The notable exception among the strong-response strains was AU/SsJ (Fig. 6, lane C), which exhibited the sequence variations associated with the reduced-response strains in the region of nuclease hypersensitivity, which include the 22-bp deletion and the transversion at residue 7973.

The previous designation of the AU/SsJ strain as Gus^{a} (38) may have been inappropriate, as indicated by additional data pertaining to the structure and androgen response of this strain (36a). The electrophoretic mobility of the AU/SsJ enzyme is characteristic of that of Gus^{b} and Gus^{h} strains. While exhibiting the strong response characteristic of Gus^{a} strains, AU/SsJ displays the 2-day lag characteristic of Gus^{b} and Gus^{h} strains. These features of AU/SsJ suggest that the control of the androgen response of GUS may be dissected into at least two components: the extent of the response and the length of the lag prior to the response. Changes within

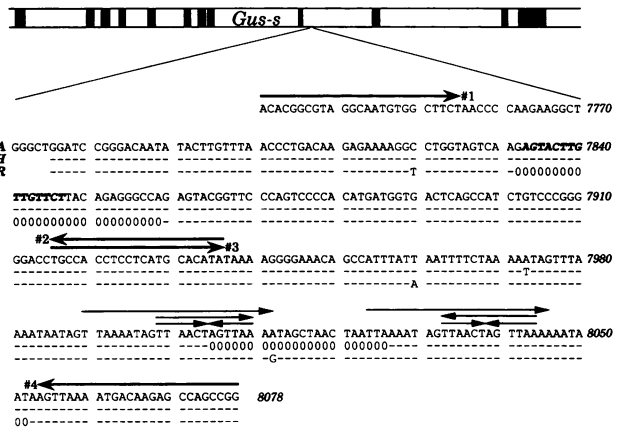

FIG. 5. Comparison of DNA sequence from inbred strains representing three major GUS androgen response phenotypes. The GUS structural gene is depicted as in Fig. 1. The expanded area represents the region of intron 9 examined by PCR and DNA sequencing. Sequence numbering commences at the transcription initiation site of *Gus-s*, as described by D'Amore et al. (13). Dashes indicate nucleotide identities with those of the published Gus^a DNA sequence (13), and a zero indicates that a nucleotide is missing relative to the Gus^a sequence. An area of complex dyad symmetry is depicted by arrows between residues 7990 and 8046. The 23-bp direct repeat is indicated by the two long arrows (7990 to 8012 and 8024 to 8046); the 12-bp inverted repeat, contained within the direct repeat, is designated by the arrows spanning positions 7999 to 8010 and 8033 to 8044. Palindromic sequences within this inverted repeat are indicated by the adjacent arrows at positions 7999 to 8004 and 8005 to 8010 and again at positions 8033 to 8038 ad 8039 to 8044. A consensus GRE sequence is indicated in bold type and spans positions 7833 to 7847. PCR in the presence of [α-^{32}P]dATP was used to generate four probes with use of the indicated primers: A4, primers 1 and 4; A4a and OR4a, primers 3 and 4; and A4b, primers 1 and 2. The Gus^a sequence, labeled A and representing the strong-response strains, was verified by sequencing 17 subclones representing seven PCR reactions from spleen DNA (obtained commercially from the Jackson Laboratory) for BALB/cJ, DBA/2Ha, and A/J mice. Sequences of the reduced-response haplotypes, Gus^b and Gus^h, labeled B/H, were identical. The B/H sequence was determined from four subclones representing two PCR reactions from spleen DNA (obtained commercially from the Jackson Laboratory) of C57BL/6J, a Gus^b strain, and of C3H/HeJ, a Gus^h strain. The sequence of the nonresponding Gus^{or} haplotype, designated OR, was determined from 11 subclones representing five PCR reactions of spleen DNA prepared from *Mus hortulanus* mice obtained from Verne Chapman, Roswell Park Cancer Institute, Buffalo, N.Y.

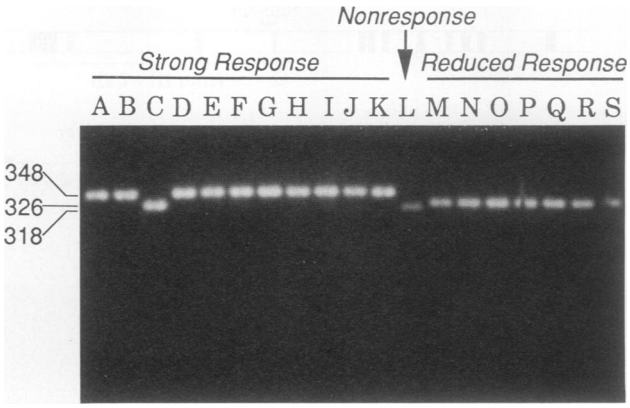

FIG. 6. Electrophoretic profile of PCR products from a series of inbred mouse strains. PCR (primers 1 and 4 in Fig. 5) was used to amplify a segment of DNA from intron 9 of *Gus-s*. Five microliters of the PCR reaction mix was subjected to electrophoresis through 2% agarose in 1× TBE (0.09 M Tris-borate [pH 8.3], 0.001 M EDTA). Sizes of the products are indicated in base pairs at the left. Inbred strains examined are as follows: lane A, A/J; lane B, SM/J; lane C, AU/SsJ; lane D, HRS/J; lane E, SEA/GnJ; lane F, DBA/2Ha; lane G, BALB/cJ; lane H, W-5; lane I, W-14; lane J, W-16; lane K, W-17; lane L, *M. hortulanus*; lane M, DBA/2J; lane N, C57BL/6J; lane O, YBR/Ei; lane P, W-12; lane Q, CBA/J; lane R, AKR/J; lane S, C3H/HeJ. Strains designated by the prefix W were constructed, and the DNA was generously donated by Verne Chapman, Roswell Park Cancer Institute, Buffalo, N.Y. Each is a congenic strain carrying the GUS gene complex of the following mouse strains on a C57BL/6J inbred background: W-5, MOR strain; and W-14, PAC strain. W-12, W-16, and W-17 represent the GUS complex of three strains originating from feral mice isolated in southern Jutland by J. T. Nielson, University of Aarhus, Denmark (12). All other DNAs were obtained from the Jackson Laboratory. Strains were grouped into those exhibiting either a strong or a reduced response of kidney GUS to androgen, with the groups being separated by the one nonresponse strain (30, 38, 40).

and surrounding the DNA region in intron 9 identified in these studies may modulate the lag and/or the extent of the kidney GUS response.

W-12 (Fig. 6, lane P) represents a GUS congenic strain derived from feral Danish mice which exhibits an apparent recombinant phenotype (12). Although Gus^a-like with respect to GUS enzyme structure, W-12 exhibits a reduced, or Gus^b-like, androgen response, along with the sequence variations associated with the reduced-response strains in the region of nuclease hypersensitivity. Another GUS congenic strain, W-17 (12) (lane K), which is also derived from feral Danish mice, exhibits a Gus^a-like enzyme structure, a strong androgen response, and an intact region of dyad symmetry. Therefore, with the noted exception of the AU/SsJ strain, the strong response of GUS to androgen is associated with an intact region of dyad symmetry, whereas the reduced response is observed in animals with the 22-bp deletion and the transversion at residue 7973.

An androgen-stimulated factor(s) in kidney extracts binds to that region of intron 9 containing DH site 7.9. If the region in intron 9 identified by DNase I hypersensitivity (DH site 7.9) contains a *cis*-active androgen response element, this element should bind a kidney factor (or factors) necessary for regulating the androgen response. To test this prediction, a gel shift comparison of a 348-bp probe (A4) from within the region containing DH site 7.9 was made between kidney nuclear extracts from androgen-treated and untreated Gus^a mice.

The results (Fig. 7) revealed two specific bands in tests of kidney extracts from androgen-treated mice, one of which was absent (or present at very low levels) when kidney extracts of untreated mice were tested. The other, while clearly present in extracts of untreated mice, was significantly increased upon treatment with androgen. To test the specificity of binding, unlabeled competitor DNA, prepared from the same PCR template as the radiolabeled probe in the molar ratios shown on the figure, was combined with extract prior to the addition of the radiolabeled probe. The intensity of a third shifted band was not reduced by the same increasing levels of unlabeled probe and therefore appeared to represent nonspecific binding. To control for possible sequence errors introduced during the PCR amplification, the results were verified several times, using independent PCR products derived from a Gus^a genomic clone template.

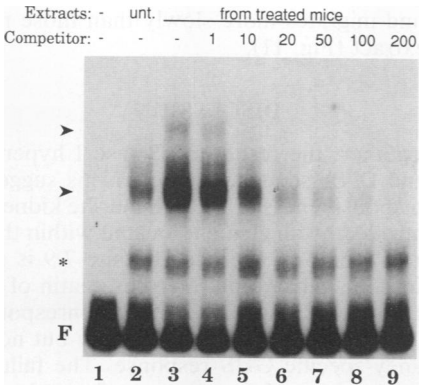

FIG. 7. Gel shift analysis of a 348-bp *Gus*^{a} DNA fragment (A4) after incubation with extracts of kidney nuclei from androgen-treated and untreated mice. Lanes: 1, free probe; 2, probe incubated with kidney nuclear extract from untreated (unt.) mice of the A/J strain; 3, probe incubated with kidney nuclear extract from A/J mice treated with androgen for 14 days; 4 to 9, same as lane 3 but with increasing molar ratios of competitor to probe, as indicated. Designated gel bands: F, free probe; *, nonspecifically bound probe; arrowhead, specifically bound probe.

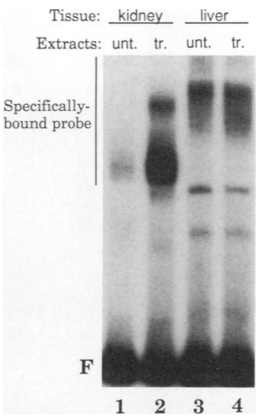

FIG. 9. Gel shift analysis of a 161-bp *Gus*^{a} DNA fragment (A4a) after incubation with extracts of kidney and liver nuclei from androgen-treated and untreated *Gus*^{a} mice. Lanes: 1, probe incubated with kidney nuclear extract from untreated mice of the A/J strain; 2, probe incubated with kidney nuclear extract from A/J mice treated with androgen for 7 days; 3, probe incubated with liver nuclear extract from untreated mice of the A/J strain; 4, probe incubated with liver nuclear extract from A/J mice treated with androgen for 7 days. Free probe is designated F.

To delineate more precisely the region of DNA responsible for the formation of the two specific bands in tests of kidney extracts from androgen-treated mice, the 348-bp (A4) probe was dissected into two parts. PCR primer pairs were designed which generate a 5′ fragment of 187 bp (A4b), which encompasses the consensus GRE sequence, as well as a 3′ fragment of 161 bp (A4a), which includes the complex repeat region (Fig. 5). Although a very weak band was observed with the A4b fragment, the two specific bands observed with the 348-bp fragment shown in Fig. 7 were also seen when the A4a probe was used in gel shift assays (Fig. 8), thus narrowing the region of binding to the 161-bp fragment which contains the region of complex dyad symmetry. Liver extracts did not produce the same shifted bands observed with kidney extracts, but rather generated two differently shifted specific bands which exhibited similar levels in both treated and untreated mice (Fig. 9).

Experiments were then performed to determine whether the region of complex dyad symmetry was capable of specifically binding the kidney factor(s). Increasing amounts of a 57-bp fragment (corresponding to the complex repeat region) and the 161-bp A4a fragment were used as competitors in gel shift assays of radiolabeled A4a incubated with kidney nuclear extracts from androgen-treated mice. As shown in Fig. 10, the 57-bp fragment competed as efficiently as A4a for the kidney binding factor(s). This result suggests

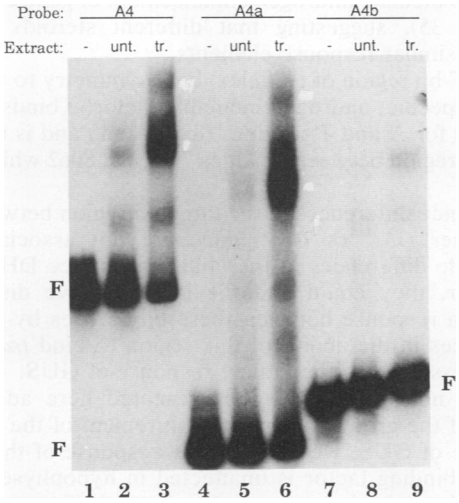

FIG. 8. Gel shift analysis of A4, A4a, and A4b probes after incubation with extracts of kidney nuclei from androgen-treated and untreated *Gus*^{a} mice. Lanes: 1, 4, and 7, free A4, A4a, and A4b probes, respectively; 2, 5, and 8, A4, A4a, and A4b probes, respectively, incubated with kidney nuclear extract from untreated mice of the A/J strain; 3, 6, and 9, A4, A4a, and A4b probes, respectively, incubated with kidney nuclear extract from A/J mice treated with androgen for 7 days. Free probe is designated F.

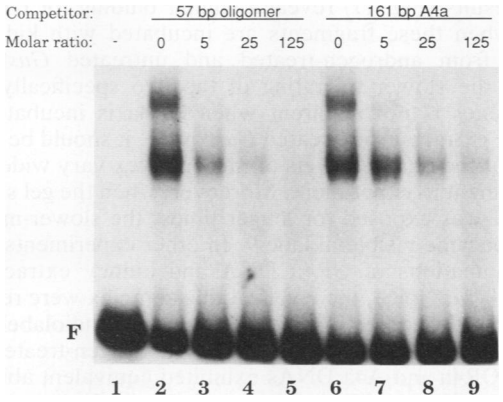

FIG. 10. Gel shift analysis of a 161-bp *Gus*^{a} fragment (A4a) after incubation with an extract of kidney nuclei from androgen-treated *Gus*^{a} mice. Lanes: 1, free probe; 2 and 6, probe incubated with kidney nuclear extract from androgen-treated A/J mice; 3 to 5, same as lane 2 but with increasing molar ratios of competitor (57-bp double-stranded oligomer) to probe, as indicated; 7 to 9, same as lane 2 but with increasing molar ratios of competitor (A4a) to probe, as indicated. Free probe is designated F.

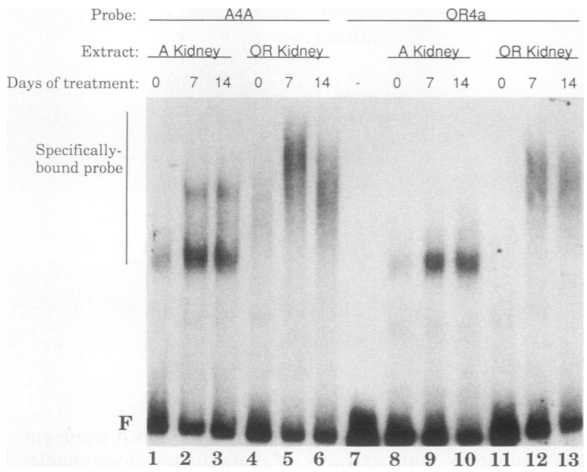

FIG. 11. Gel shift analysis of a 161-bp *Gus*^{a} DNA fragment (A4a) or a 159-bp [*Gus*]^{or} fragment (OR4a) after incubation with extracts of kidney nuclei from androgen-treated and untreated *Gus*^{a} or *Gus*^{or} mice. Lanes: 1 to 3, A4a probe incubated with kidney nuclear extracts from A/J mice treated with androgen for 0, 7, or 14 days, respectively; 4 to 6, A4a probe incubated with kidney nuclear extracts from *M. hortulanus* mice treated with androgen for 0, 7, or 14 days, respectively; 7, free OR4a probe; 8 to 10, OR4a probe incubated with kidney nuclear extract from A/J mice treated with androgen for 0, 7, or 14 days, respectively; 11 to 13, OR4a probe incubated with kidney nuclear extracts from *M. hortulanus* mice treated with androgen for 0, 7, or 14 days, respectively. Free probe is designated F.

that the band shifts observed with kidney extracts represent binding to sequences within the 57-bp region of complex dyad symmetry.

Since the failure of kidney GUS to respond to androgen in *Gus*^{or} mice is under the control of a *cis*-acting element, it is possible that this failure is due to the inability of *Gus*^{or} DNA to bind the inducible kidney factor. Gel shift assays were performed to determine whether the analogous DNA fragment of *Gus*^{or} mice (OR4a) displays a similar gel binding pattern when incubated with kidney extracts of *Gus*^{a} mice. The results (Fig. 11) reveal a similar binding for OR4a and A4a when these fragments are incubated with kidney extracts from androgen-treated and untreated *Gus*^{a} mice. While the slower migrating of the two specifically bound complexes is not apparent when OR4a is incubated with kidney extracts from treated *Gus*^{a} mice, it should be pointed out that the relative levels of this complex vary widely from experiment to experiment. Moreover, when the gel shown in Fig. 11 was exposed for longer times, the slower-migrating band became visible in lane 9. In other experiments involving incubations of OR4a DNA and kidney extracts from treated *Gus*^{a} mice, the levels of this complex were relatively high. Furthermore, in binding studies of radiolabeled A4a DNA with kidney extracts from androgen-treated *Gus*^{a} mice, OR4a and A4a DNAs exhibited equivalent abilities to compete with the labeled probe (data not shown).

Even though the failure of GUS to respond in OR mice is controlled by a *cis*-acting element, we nevertheless tested kidney nuclei of OR mice for the presence of the binding factor observed in A mice. Incubation of A4a or OR4a with extracts from androgen-treated and untreated mice of the *Gus*^{or} haplotype reveals a specific band which is significantly increased upon treatment with androgen. However, the band is diffuse and migrates more slowly than those revealed by the *Gus*^{a} extract (Fig. 11).

DISCUSSION

Taken together, the results of DNase I hypersensitivity, gel shift, and DNA sequence comparisons suggest that the androgen responsiveness of GUS in murine kidney epithelial cells is controlled by an element located within the proximal end of intron 9 of *Gus-s*. Since DH site 7.9 is induced by androgen in both kidney and liver chromatin of responsive mice but is absent in treated mice of the nonresponsive *Gus*^{or} haplotype, its presence may be necessary but not sufficient for the kidney-specific GUS response. The failure of liver GUS activity to respond to androgen after induction of DH site 7.9 could result from the absence or exclusion of the necessary binding factor(s) which regulates this response in kidney. The observation that a DH site, such as that seen in liver of androgen-treated female mice, may be necessary but not sufficient for enhancement has precedence in results described for the tyrosine aminotransferase gene (32). While glucocorticoid enhancement of expression of this gene is accompanied by the induction of a strong DH site at −2.5 kb in most examined hepatoma cell lines and in liver, one hepatoma line exhibits only the DH site induction and not the enhancement.

A comparison of the gel shift patterns of DNA fragments which exhibit DH site 7.9 after incubation with kidney and liver extracts suggests the presence of one or more androgen-inducible factors which may be directly involved in the response of kidney GUS. If so, then the *cis*-active failure of kidney GUS to respond to androgen in *Gus*^{or} mice (30) could result from an inaccessibility of the kidney-specific factor(s) to DNA, as reflected in the absence of DH site 7.9 in *Gus*^{or} chromatin. This failure to induce DH site 7.9 could, in turn, be a consequence of the 28-bp deletion in *Gus*^{or} DNA which removes the GRE-like sequence shown in Fig. 5. GREs present in the tyrosine aminotransferase gene and the long terminal repeat of mouse mammary tumor virus have been shown to mediate androgen enhancement of gene expression (14, 25, 35), suggesting that different steroids may act through similar response elements.

The 57-bp region of complex dyad symmetry to which the kidney-specific, androgen-inducible factor(s) binds is highly enriched for A and T residues (82% A+T) and is contained within a region between residues 7954 and 8062 which is 87% A+T.

Sequence differences in the targeted region between *Gus*^{a} and either *Gus*^{b} or *Gus*^{h} mice are not associated with detectable differences in the ability to induce DH site 7.9. However, they could underlie the *cis*-active disparity of androgen response between these haplotypes by imparting differences in the ability of this region to bind *trans*-acting factors essential to the kidney response of GUS.

While none of the results presented here address the nature of the growth hormone requirement of the androgen response of GUS, we find that the response of the kidney-specific binding factor is unaffected in hypophysectomized mice and in mice homozygous for the *dwarf* mutation (39a).

Hormone-inducible DH sites have been identified for several steroid hormone-responsive genes (6, 7, 22, 23, 44). These studies have contributed to the identification of hormone response elements for glucocorticoids, progesterones, and estrogens (1, 31, 34). Identification of response elements for androgens has been complicated by the presence of overlapping GREs and/or progesterone response elements

among those androgen-regulated genes which have been compared (8, 9, 24, 25). For example, comparisons of the 5′ flanking sequences of androgen-regulated genes have shown some sequence similarity, but not enough to generate a consensus (28, 42). Closely associated with the androgen-inducible DH site 7.9 identified in our study is a GRE-like sequence in DNAs of the androgen-responsive A, B, and H haplotypes but not in DNA of the nonresponsive OR haplotype. Since such sequences are known to mediate the androgen response (25) and have been associated with regions of DNase I hypersensitivity (44), it is possible that the GRE-like sequence located within intron 9 of *Gus-s* interacts with the androgen receptor to generate the accessibility needed for the androgen-inducible binding factor(s) in kidney to bind and mediate the response of GUS in kidney epithelial cells. Others have shown that the specificity of the response may require cell-specific factors in addition to the androgen receptor (24, 39).

The majority of evidence to date suggests that the primary effect of androgens is at the level of gene transcription (5, 10, 14, 15, 25, 35, 41) and is mediated by the interaction of a steroid hormone-receptor complex and a *cis*-acting DNA sequence in the vicinity of the responding gene. The identification of yet another GRE-like sequence which may impart androgen responsivity reinforces the view that different steroids may act through similar response elements. The specificity may be due to subtle differences in the sequence of the imperfect palindrome required to elicit the hormone response or alternatively to the requirement of cell-specific factors in addition to the hormone receptor. Tests are under way in our laboratory to address these issues with respect to the murine β-glucuronidase system.

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