Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4816–4820, July 1987
Biochemistry

DNase I-hypersensitive sites associated with expression and hormonal regulation of mouse *C4* and *Slp* genes

(chromatin/androgen regulation/complement)

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Communicated by Cyrus Levinthal, March 23, 1987

ABSTRACT **There are four major regions of DNase I hypersensitivity in the 5′ regions of the genes for the murine fourth component of complement (*C4*) and its homologous neighbor, *Slp* (sex-limited protein). Hypersensitivity around the start site of transcription and ≈0.5 kilobases upstream correlates qualitatively with expression of these genes. Two hypersensitive sites, at −2.3 and −2.0 kilobases, map specifically to the *Slp* gene and correlate with its hormonal regulation. That is, these sites are more prominent in male liver chromatin and become more apparent in chromatin from females treated with testosterone. Further, these sites are established in males to a greater extent than in females prior to expression of *Slp* and may reflect gene-commitment events. Comparison of chromatin from mouse strains differing in *C4* and *Slp* alleles indicates that the four regions of hypersensitivity may be necessary but are not sufficient for high levels of expression.**

Alterations in chromatin structure detected by nuclease digestion have been shown to reflect regulatory events in gene expression (1). Localized regions of DNase I hypersensitivity are observed near the 5′ ends of active genes, as well as at internal and 3′ regions, and seem to be necessary but not sufficient for efficient transcription (2). Causal relationships between DNase I-hypersensitive (HS) sites and gene activation are not well defined. However, for some regulated genes, such as mouse mammary tumor virus, *Drosophila* heat shock, and chicken adult β-globin, specific proteins have been shown to bind in or near these HS regions (3–6). In addition, chromatin structure may indicate gene commitment events prior to actual expression, as has been shown for the chicken vitellogenin gene (7).

We are investigating two closely related genes located in the mouse major histocompatibility complex to correlate specific chromatin structures with different patterns of expression. The *C4* gene encodes the fourth component of complement whereas the product of its neighboring gene *Slp* (sex-limited protein) does not function in the complement pathway and is regulated by androgens (8). The major site of synthesis for both proteins is the liver. Congenic strains of mice carry alleles for *C4* and *Slp* that differ in their expression. The three major *Slp* alleles are androgen-regulated (Slp^a), constitutive (Slp^c), and null (Slp^o) (9). *C4* expression can differ 20-fold, determined by *C4*-high ($C4^h$) or *C4*-low ($C4^l$) alleles (8). Molecular analyses of cDNA clones from various strains indicate that the coding sequences for C4 and Slp are greater than 95% homologous (10–13). Molecular mapping of genomic clones shows that both genes are ≈16 kilobases (kb) long, are transcribed in the same orientation, and result from an apparent tandem duplication of ≈50 kb of chromosomal DNA around the *C4* gene (14). Subsequent divergence has resulted in the striking functional and regulatory differences between C4 and Slp.

This system is conducive to analysis of chromatin structures associated with developmental, hormonal, and tissue-specific regulation because of the ability to compare *C4* to *Slp* and to compare alleles in various mouse strains. We have examined hypersensitivity to DNase I in the 5′-flanking regions of these genes. Two far upstream HS sites map specifically to the *Slp* gene and reflect *Slp* expression qualitatively but not quantitatively. Similarly, HS sites nearer the promoters are not sufficient for high levels of expression of *C4* or *Slp*.

MATERIALS AND METHODS

Materials. Restriction and modification enzymes were purchased from New England Biolabs and DNase I (DPFF) from Worthington. [α-^{32}P]dCTP was from New England Nuclear. Testosterone proprionate was from Sigma, and digitonin was from Fluka.

Animals and Hormonal Treatment. B10.D2 ($H\text{-}2^d,C4^h,Slp^a$) and B10.BR ($H\text{-}2^k,C4^l,Slp^o$) mice, originally from The Jackson Laboratory, and B10.W7R ($H\text{-}2^{w7},C4^h,Slp^c$) mice, obtained from A. Ferreira and V. Nussenzweig (New York University School of Medicine), are maintained in our animal facility. For hormonal induction, adult female mice were injected three times per week for 2 weeks with 2.5 mg of testosterone proprionate in 0.1 ml of sesame oil.

Isolation of Nuclei and DNase I Digestion. Nuclei from fresh tissues were isolated according to Burch and Weintraub (7) and Fritton *et al.* (15). For most points, three sets of two or three mice were analyzed. Tissues were minced on ice, washed with Dulbecco's phosphate-buffered saline, and homogenized in 15 ml/g of HB1 (0.5 M sucrose/0.5 mM spermine/0.15 mM spermidine/60 mM KCl/150 mM NaCl/15 mM Tris·HCl, pH 7.4/0.2 mM EDTA/0.2 mM EGTA/1 mM phenylmethylsulfonyl fluoride/0.1% digitonin). Nuclei were pelleted by centrifuging for 10 min in a clinical centrifuge and washed twice in HB2 (HB1 without digitonin and with 0.35 M sucrose). The nuclear pellet was suspended in HB3 (HB1 without digitonin and sucrose), at about 1.5 mg of DNA per ml, and distributed in aliquots, and DNase I was added to 0, 60, 90, 180, 220, 290, and 360 units/ml. Samples were preincubated at 37°C for 3 min, and the 5-min digestion was initiated by addition of $MgCl_2$ to 5 mM. EDTA was then added to 10 mM, and nuclei were pelleted briefly and washed once in HB4 (HB1 without sucrose, digitonin, and phenylmethylsulfonyl fluoride). Final nuclear pellets were resuspended in HB4 and extracted with proteinase K at 200 μg/ml for 16 hr at 25°C and then extracted with phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol), and with chloroform/isoamyl alcohol, 24:1 (vol/vol), and precipitated with etha-

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Abbreviation: HS, hypersensitive.

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nol. DNA was resuspended in 10 mM Tris·HCl, pH 8.0/1 mM EDTA (TE), digested with RNase A at 50 μg/ml for 30 min at 37°C, extracted, and precipitated as above. Final pellets were resuspended in TE. Control protein-free liver DNA was digested with DNase I at 0–16 units/ml for 10 min at 37°C in HB1, digested with proteinase K, extracted, and precipitated as above.

Southern Blotting. Restriction digests contained 15 μg of genomic DNA and 30 units of the appropriate enzymes. Samples were electrophoresed on either horizontal 1.2% agarose gels (20 cm × 6 mm) overnight at room temperature or on vertical 1.6% agarose gels (40 cm × 4 mm) for 48–72 hr at 4°C. Gels were electroblotted onto GeneScreen*Plus* (New England Nuclear), and the filters were prehybridized, hybridized, and washed according to the supplier's instructions. Probes were nick-translated (16) to 10^9 cpm/μg, and 3–6 × 10^7 cpm were hybridized to blots in minimal volume (20 μl/cm² of filter). Autoradiograms were scanned using an LKB UltroScan XL densitometer.

RESULTS

Mapping of HS Sites in the 5′-Flanking Regions of *C4* and *Slp*. We compared male and female HS sites of the *C4* and *Slp* genes for differences that might correlate with androgen regulation of Slp. In the B10.D2 strain, male mice express Slp only after puberty; females express Slp at 1% adult male levels, also only after puberty (17). Both sexes express high levels of C4; adult males show twice the level of C4 as females, as is found for all *C4* alleles (8). Mapping of HS sites for these genes is complicated by their strong homology. As shown in Fig. 1*A*, *Kpn* I sites in the 5′-flanking regions of these genes differ, resulting in 9.5-kb and 4.6-kb *Kpn* I fragments spanning the transcription start sites for *Slp* and *C4*, respectively. We used indirect end-labeling (18) with a 0.6-kb *Pvu* II–*Bgl* II fragment of a *C4* genomic clone to map HS sites within these *Kpn* I fragments. This probe is ≈0.6 kb upstream of the 3′ *Kpn* I site common to both *Slp* and *C4*.

The HS sites in chromatin from B10.D2 mice are shown in Fig. 1*B*. Samples compared in this figure, as in all others, correspond to points that showed the same extent of DNase I digestion in Southern blots of previous DNase I titration curves. Optimal digestion was usually at 180–220 units of DNase I per ml. Extents of DNase I digestion were confirmed to be comparable by reprobing blots with a 5′-flanking fragment of the mouse albumin gene, which does not show a sex difference in expression. The 7.0-kb *Kpn* I albumin band shows three HS sites that are the same intensity in male and female liver chromatin (Fig. 1*C*). These sites were used as internal controls for extent of digestion of different liver samples. In this study, variation in digestion between compared samples was never >20%, as judged by densitometric scanning of blots reprobed with albumin DNA.

The most prominent HS sites appear below the 4.6-kb *C4* band and thus could be derived from either *C4*, or *Slp*, or both. In male liver chromatin, there are four major regions of hypersensitivity that map within the 4.6 kb upstream of the 3′ *Kpn* I site common to both *Slp* and *C4* fragments. Regions 1, 2, and 3 correspond to approximately −2.3 kb, −2.0 kb, and −0.5 kb from the transcription start site, respectively. The band at region 3 appears to be a doublet (see Fig. 3). Region 4 can be resolved further (see Fig. 3) and contains a minimum of five bands ranging from near the start site of transcription to approximately −0.25 kb. Longer autoradiographic exposures reveal weaker sites at −1.8 kb and immediately downstream from the start site.

A similar set of HS sites is observed in female liver chromatin. These sites, however, exhibit markedly lower intensity than in the male, especially at HS sites 1 and 2. Reduction of HS sites 3 and 4 in female chromatin may be indicative in part of one active gene (*C4*) rather than two (*C4* and *Slp*). Sites that migrate below the 9.5-kb *Slp* band and above the 4.6-kb *C4* band must be derived from *Slp* but not *C4*. Although there is one prominent HS site that migrates just below the 9.5-kb *Slp* band, it does not exhibit an obvious male–female difference. This distant site could be derived from an as yet unidentified gene upstream of *Slp*.

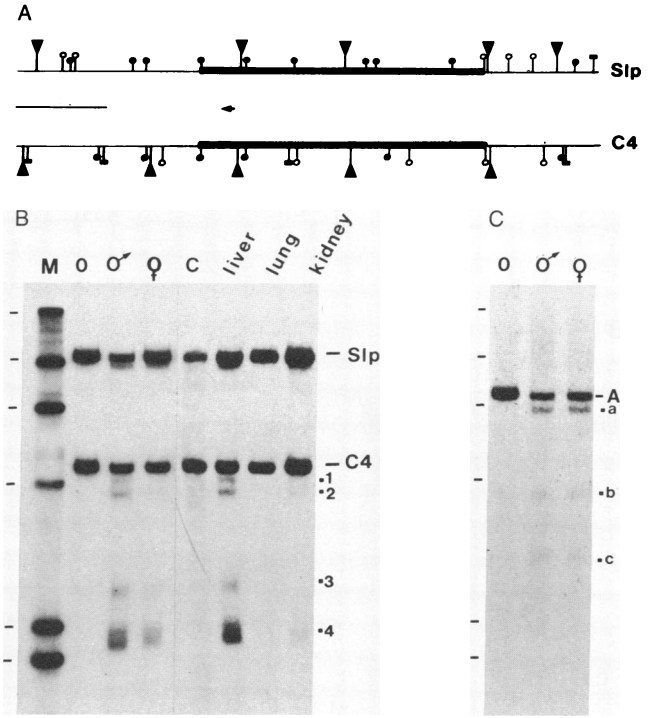

FIG. 1. DNase I HS sites in the 5′-flanking regions of *Slp* and *C4*. (*A*) Restriction maps of *Slp* and *C4* genes were derived from overlapping λEMBL3 clones from a B10.D2 library. Gene extents are shown by heavy black lines; transcription is left to right. The 600-base-pair probe is indicated by an arrow between the maps. The horizontal line at left is 5 kb. Restriction sites are *Kpn* I (▼), *Bam*HI (●), *Eco*RI (■), and *Hin*dIII (○). (*B*) DNA (15 μg per lane) was digested with *Kpn* I, electrophoresed on a 1.2% agarose gel, transferred to GeneScreen*Plus*, and hybridized to the nick-translated probe. Lane M contains *Hin*dIII-digested λ DNA (fragments are 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb). Lanes: 0, DNA from male liver nuclei incubated without DNase I; C, protein-free male liver DNA digested with DNase I (2 units/ml). Other lanes (left to right) are DNA from DNase I-digested nuclei of male (♂) and female (♀) liver, and male liver, lung, and kidney. Each sample (and those of subsequent figures) showed similar extent of digestion in DNase I titration curves; optimal digestion range was 180–220 units/ml. The 9.5-kb *Slp* and 4.6-kb *C4 Kpn* I fragments not cleaved by DNase I are marked. Bands generated by DNase I are numbered 1–4. (*C*) The filter was reprobed with a 5′-flanking fragment of the mouse albumin (A) gene; albumin HS fragments are labeled a, b, and c. Lanes are labeled as in *B*.

These HS sites are related to the tissue-specific expression of *Slp* and *C4*. They are absent in both DNase I-digested protein-free liver DNA and male lung chromatin (Fig. 1*B*). C4 and Slp are not expressed in lung, as shown by RNA analysis (data not shown). We expected kidney to be an additional nonexpressing tissue and were surprised to observe a set of HS sites from male kidney chromatin similar to liver sites. In subsequent RNA gel blot analysis, male kidney was shown to express Slp and C4 at ≈10% the level in liver (data not shown).

HS sites 1 and 2 can be assigned specifically to the *Slp* gene by digesting with both *Kpn* I and *Hin*dIII. *C4* has a *Hin*dIII site about 0.5 kb downstream from the 5′ end of the 4.6-kb *Kpn* I fragment (Fig. 2*A*). *Slp* lacks this *Hin*dIII site and has

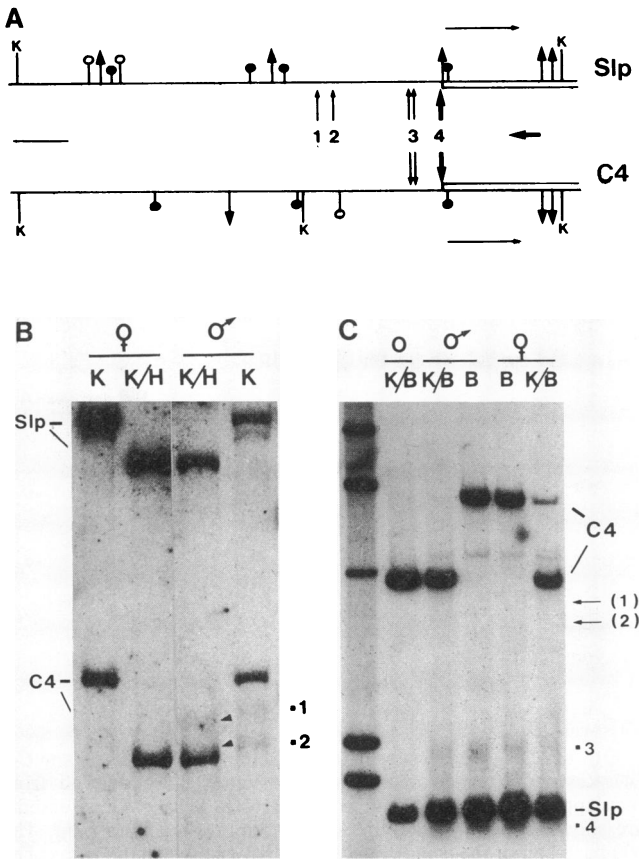

FIG. 2. Mapping of HS sites 1 and 2 to the *Slp* gene. (*A*) The 5′ fragments of *C4* and *Slp* are shown with sites for *Bam*HI (●), *Bgl* II (▲), *Hin*dIII (○), and *Kpn* I (K). Transcription is noted by rightward arrows, and the probe is noted by a leftward arrow. The horizontal line at left is 1 kb. HS regions 1–4 are shown by vertical arrows reflecting data described. (*B*) DNA from DNase I-treated male (♂) and female (♀) nuclei was digested with *Kpn* I (K) or *Kpn* I and *Hin*dIII (K/H) and electrophoresed on a 40-cm vertical 1.6% agarose gel. The parent 9.5-kb *Kpn* I and 8.1-kb *Kpn* I–*Hin*dIII *Slp* bands are marked, as are 4.6-kb *Kpn* I and 4.1-kb *Kpn* I–*Hin*dIII *C4* bands. HS sites 1 and 2 are noted between male lanes. (*C*) DNase I-digested liver chromatin DNAs were digested with *Bgl* II (B) or *Kpn* I and *Bgl* II (K/B) and electrophoresed on a horizontal 1.2% agarose gel. The first lane to the left is *Hin*dIII-digested λ DNA. Lane O is DNA from male liver nuclei incubated without DNase I and digested with *Kpn* I and *Bgl* II. Parent 6.0-kb *Bgl* II and 4.4-kb *Kpn* I–*Bgl* II *C4* fragments are indicated, as is the 1.7-kb *Bgl* II *Slp* fragment. HS regions 1–4 are marked. HS region 4 appears within and below the parent *Slp* band.

one further upstream. Thus, if HS sites 1 and 2 are derived from *Slp* and are further than 4.1 kb upstream of the 3′ *Kpn* I site, they should migrate above the 4.1-kb *C4 Kpn* I–*Hin*dIII band. Fig. 2*B* illustrates that both HS sites 1 and 2 migrate above the *C4* parent band, although HS site 2 is barely resolved. To show that these sites are not present in the *C4* gene, a digest was used that distinguishes *C4* from *Slp* in this region. *Bgl* II digestions of DNA from DNase I-treated nuclei do not exhibit HS sites 1 and 2 in the expected area of the gel beneath the *C4* parent band (Fig. 2*C*), even on longer autoradiographic exposure. Therefore, HS sites 1 and 2 are derived from the *Slp* gene and correlate with *Slp* expression. HS sites in regions 3 and 4 have not yet been assigned to *C4* and/or *Slp*, but both genes are likely to show hypersensitivity in these regions close to the promoters. Thus, we pursued analysis of HS sites 1 and 2 as they map specifically to the *Slp* gene (in a region that diverges from *C4* in sequence; ref. 19 and unpublished data) and may be relevant to differential regulation of the two genes.

Hormonally-Regulated Expression of *Slp* Is Reflected by HS Sites 1 and 2. To analyze whether these HS sites might be associated with hormonally regulated expression of *Slp*, liver chromatin from testosterone-treated females, females treated and then withdrawn from hormone, and castrated males was analyzed (Fig. 3). The HS pattern qualitatively typical of adult males can be induced in females by treatment with androgen. Female expression of Slp rises to about half that of the male after such induction (ref. 20; unpublished data). Liver chromatin from treated females withdrawn from hormone for 19 days exhibits similar hypersensitivity to chromatin from normal females; that is, all HS sites are significantly reduced. In contrast, chromatin from males castrated 2 weeks prior to sacrifice does not show such a marked decrease in hypersensitivity at HS site 2, whereas HS sites 1, 3, and 4 are reduced more noticeably. For both males and females, withdrawal from testosterone for this length of time decreases the Slp levels substantially but does not completely extinguish expression (ref. 20; unpublished data).

To attempt a more quantitative comparison and account for minor variation in the amounts of DNA per lane, autoradiograms were analyzed by scanning densitometry, and the relative peak areas for HS regions of Fig. 3 were calculated. The ratio of the male *C4* parent band area to the *C4* band area of a given sample was used to adjust HS site areas for direct comparison. Hypersensitivity in regions 1–4 is 2.5- to 4-fold greater in male than female chromatin. Females treated with testosterone exhibit approximately doubled hypersensitivity at all four sites. The scans emphasize that the HS sites at the extremes of region 4 show greater response to hormone than the central portion, but these sites have not been individually mapped to *Slp* or *C4*. Quantitative analysis confirms the qualitative description of the autoradiograms but is subject to limitations. For example, as the peaks of HS sites 1 and 2 overlap in the scans and as HS site 1 occurs on the shoulder of the *C4* parent band, the areas are difficult to define. Furthermore, whereas males and females differ in hypersensitivity several-fold, *Slp* expression differs 100-fold and, therefore, relatively minor apparent changes in chromatin structure may be associated with large changes in transcription.

HS Sites Are Established Prior to *Slp* Expression. Slp expression in mice carrying the androgen-regulated allele is first detected around the time of puberty, at about 6 weeks of age (9). Neither males nor females can be treated so as to express Slp prior to this time, implying that other factors or hormones may be required in addition to increased androgen levels. Alternatively, levels of the androgen receptor may be limiting. To assess whether the *Slp* gene shows evidence of commitment, we examined HS sites in liver chromatin prior to expression of *Slp* (Fig. 3). Hypersensitivity at site 2 is consistently greater in males than in females at all ages examined. Densitometry indicates that at 4 weeks of age, male and female HS patterns are quantitatively the same as in adults even though Slp expression is not yet detected. At 2 weeks, the HS patterns are more similar to the adult female for both sexes, with the exception of HS site 2 that is approximately twice as intense in males as in females. Thus, it appears that hypersensitivity is gradually acquired in males to a greater extent than in females prior to expression of the *Slp* gene, especially at HS site 2.

HS Sites of *C4* and *Slp* Alleles Differing in Their Regulation. Several regulatory mutations in *C4* and *Slp* are carried in congenic mouse strains. We have examined two other strains in the B10 background to ask whether any of these cis-acting mutations are associated with different HS patterns that might then be correlated with different modes of *C4* and *Slp* expression. In B10.BR mice ($H\text{-}2^k, C4^l, Slp^o$), *C4* expression

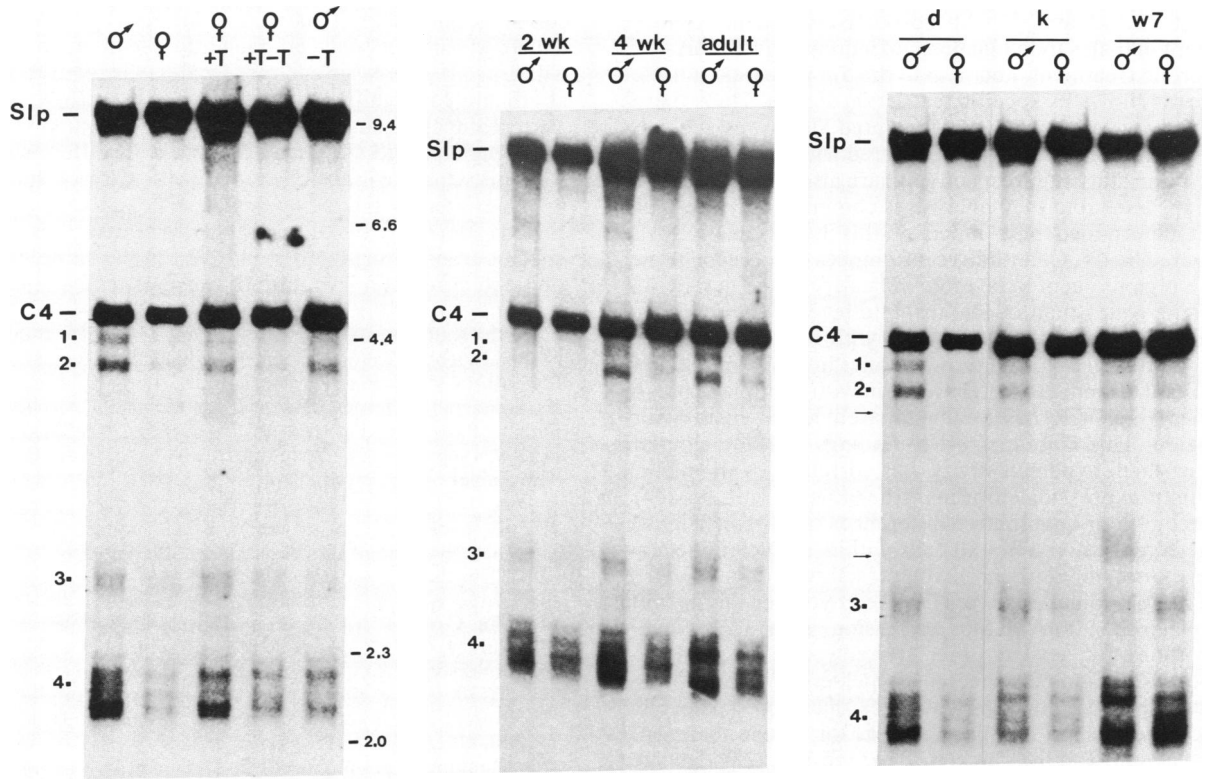

FIG. 3. (*Left*) HS sites in liver chromatin of hormonally treated mice. DNA (15 μg per lane) from DNase I-digested B10.D2 liver nuclei were fractionated on a vertical 1.6% agarose gel and Southern blotted. Chromatin was derived from the following animals: adult males (♂) and females (♀), females treated for 2 weeks with testosterone (+T), females treated for 2 weeks and then withdrawn from testosterone for 19 days (+T−T), and males castrated 2 weeks prior to sacrifice (−T). Notation at left is as in Fig. 1. Numbers at right are *Hin*dIII-digested λ DNA fragments. (*Center*) HS sites in liver chromatin of immature mice. DNA samples (15 μg) from DNase I-treated liver nuclei of B10.D2 mice were digested with *Kpn* I and electrophoresed on a vertical 1.6% agarose gel. Immature 2-week-old and 4-week-old males (♂) and females (♀) and adult males and females are compared. Notation is as in Fig. 1. (*Right*) HS sites in liver chromatin of mice with different *Slp* and *C4* alleles. Lanes: d, k, and w7, DNase I-digested chromatin from B10.D2 ($H\text{-}2^d$, $C4^h$, Slp^a), B10.BR ($H\text{-}2^k$, $C4^l$, Slp^o), and B10.W7R ($H\text{-}2^{w7}$, $C4^h$, Slp^c) male (♂) and female (♀) mice, respectively. DNAs were digested with *Kpn* I and Southern blotted. Additional HS regions in B10.W7R mice are indicated by arrows.

in liver is 5% that of strains carrying the $C4^h$ allele. Although characterized as a null expressor of *Slp*, this strain does show extremely low levels of Slp in males (21). The B10.W7R mouse ($H\text{-}2^{w7}$,$C4^h$,Slp^c) is a high expressor of C4 and constitutively expresses Slp in both sexes (9).

A comparison of DNase I-digested liver nuclei from these strains is shown in Fig. 3. *Kpn* I sites in the 5′ regions of the *C4* and *Slp* genes in B10.BR DNA are the same as in B10.D2. The HS sites are remarkably similar as well, despite the greatly reduced levels of expression of both genes in B10.BR mice (k haplotype). That is, HS sites 1–4 are more prominent in male than in female chromatin and are similar in intensity to the sites in the B10.D2 strain (d haplotype). This implies that DNA sequences or chromatin structure necessary for formation of these sites are similar in the B10.BR strain and again may reflect hormonal regulation of *Slp* expression. However, these sites in themselves are not sufficient to ensure high levels of expression. HS site 4 may be somewhat less but certainly not in accord with a 20 times reduction of *C4* expression and an ≈100 times reduction of *Slp* expression compared to B10.D2 mice.

There are four distinguishable Slp^{w7} genes in the B10.W7R strain (22, 23, 27). One has a 9.5-kb *Kpn* I fragment like Slp^d and the other three (and the single $C4^{w7}$) have 4.6-kb *Kpn* I fragments like $C4^d$. The chromatin digestion pattern is somewhat similar to that of B10.D2 mice, with increased hypersensitivity at regions 3 and 4 possibly indicating the greater number of expressing genes in B10.W7R mice. HS site 2 is distinct in both males and females of the B10.W7R strain, whereas HS site 1 is more pronounced in males but less intense than in the B10.D2 male. More notable differences between this strain and the others include a HS region at −1.0 kb that is present in males but not females and an enhanced site at −1.8 kb that is minor in the other strains. Further analysis will determine which of the five *C4*/*Slp* genes in this strain account for hypersensitivity at site 2, and whether this reflects tissue-specific expression of *Slp* that in some strains is also dependent on androgen. The complexity of the B10.W7R HS pattern may reflect differential regulation of the multiple *Slp* genes (27).

DISCUSSION

The *C4* and *Slp* genes are very homologous but differ in aspects of their regulation. Coupled with the variety of regulatory alleles known for these genes, this provides an opportunity to correlate specific DNA sequences with differential expression. To begin to understand the effects of some of these sequences (and factors that interact with them), we have analyzed chromatin structure in the 5′-flanking gene regions. *C4* and *Slp* are accessible to this analysis as they are expressed in an abundant tissue that differentiates to primarily one cell type in which a major change in expression occurs for one of the two genes late in development (androgen induction of *Slp* expression at sexual maturation).

In this report, we describe four major regions of hypersensitivity observed upon DNase I digestion of liver chromatin from B10.D2 mice (summarized in Fig. 2*A*). HS sites

1 and 2 at −2.3 kb and −2.0 kb, respectively, are much more prominent in males than females, are increased in females by testosterone treatment, and map to the *Slp* gene (and not the *C4* gene) as shown by specific restriction digests. Thus, these sites correlate with hormonally regulated *Slp* expression. HS regions 3 and 4, at −0.5 kb and clustered within 0.25 kb of the transcription start site, respectively, are also enhanced when *Slp* is expressed, but their analysis is complicated as we have not yet determined how much of this hypersensitivity is contributed by *C4*. Quantitative differences observed in some of these regions seem to be more indicative of the number of expressing genes than of the absolute level of their expression. For example, a mouse strain with low expression of both *C4* and *Slp* (B10.BR) shows similar intensity in region 4 to the B10.D2 mouse, whereas a strain with multiple expressing genes (B10.W7R) has greater apparent hypersensitivity in this region. This is in contrast to findings with the albumin gene, in which hypersensitivity was correlated with amount of albumin expression in primary hepatocyte cultures (24). Interpretation of quantitative differences in hypersensitivity may be complicated if not all cells within a population show similar expression and chromatin structure.

The correlation of upstream DNase I hypersensitivity with hormonal regulation of *Slp* expression is in accord with observations for several other steroid-regulated genes. In some cases, regions that become hypersensitive in response to hormone correspond to sequences that bind the specific steroid receptor, as has been shown for mouse mammary tumor virus (3) and the chicken lysozyme gene (25). To date, however, most HS sites induced by hormone have not been directly correlated with receptor binding to DNA. Receptor binding may cause HS sites to appear elsewhere if the effect of such binding can be propagated along the chromosome, or if the receptor interacts with or induces the synthesis of other DNA binding proteins. As yet, we do not know whether or where the androgen receptor interacts with sequences of the *Slp* gene, but a region of ≈750 base pairs that encompasses HS sites 1 and 2 is sufficient for androgen-inducible chloramphenicol acetyltransferase gene expression in transient transfection assays (ref. 27 and unpublished data).

In addition to their correlation with transcriptional activation, some HS sites appear before gene expression and may reflect a committed state, as proposed by Burch and Weintraub (7). In the case of *Slp*, upstream HS sites are detectable in male liver at 2 weeks and attain adult intensity by 4 weeks, prior to *Slp* expression. Acquisition of hypersensitivity in development may reflect an early event that is gradually augmented in postnatal liver cell division and growth. This event may commit *Slp* to express only after puberty when androgen levels are high. Delayed expression until puberty may be due to other components that are limiting, such as androgen receptor or factors such as growth hormone (26). It is tempting to speculate that the determining event may correlate with transient fetal or neonatal androgen pulses in the male that are known to be necessary for later sexual maturation. Thus, testosterone itself may play a role in committing *Slp* to hormonal regulation. In any case, HS site 2 indicates an early commitment of *Slp* in the male to hormonally regulated expression.

Expression of *Slp* can be achieved in mature females with androgen-regulated alleles by hormone administration. In part this may indicate that factors no longer limiting after puberty in males for *Slp* expression are also not limiting in mature females. However, qualitative differences in the HS complexes may exist. That is, all four HS regions of induced female chromatin seem equivalently sensitive to androgen withdrawal, whereas in castrated males, the relative decrease at HS site 2 is significantly less than at HS sites 1, 3, and 4. The greater apparent stability of the male chromatin structure in the region of HS site 2 may reflect subtle differences in the way this structure is established or in the way it is maintained in males versus induced females. The putative cis-acting regions identified by this study may be useful in isolating specific trans-acting factors involved in the complex regulation and chromatin structure of these genes.

Martha Kalff characterized the Slp^d and $C4^d$ genes in this laboratory and Debby Walthall provided technical assistance. We thank Drs. Moses Chao, Paul Wollenzien, and Larry Chasin for helpful discussions; Dr. Shirley Tilghman for the pAlb-1 probe; and Dr. Farhang Payvar and Pat Hess for use of their LKB UltroScan. This work was supported by a grant from the National Institutes of Health (GM 31546).

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