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Genetics

Male-specific transcription initiation of the *C4-Slp* gene in mouse liver follows activation of STAT5

(liver gene expression/growth hormone secretion/complement component C4/sexual dimorphism)

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ABSTRACT **The mouse genes encoding the constitutively expressed complement component C4 and its closely related isoform C4-Slp (sex-limited protein), which is expressed only in male animals of several strains, provide a unique model to study sequence elements and trans-acting factors responsible for androgen responsiveness. Our previous studies have shown that hormonal induction of *C4-Slp* is mediated by a sex-specific pattern of growth hormone secretion. Promoter analyses *in vitro* have led to contradictory conclusions concerning the significance of *C4-Slp*-specific sequences in the 5′ flanking region. Mutant mice carrying the *H-2*^{aw18} haplotype, which is characterized by a large deletion in the *S* region covering the *C4* and *21-OHase A* genes, permit the direct *in vivo* analysis of *C4-Slp* transcription, unhindered by the presence of *C4*. Run-on analysis of transcription in liver nuclei of males and females of this strain demonstrated a 100-fold higher transcriptional activity in males, essentially determined at the transcription initiation level. The androgen dependence of transcription initiation was confirmed by run-on analysis of testosterone-treated females, where transcriptional activity started after 6 days of androgen treatment and reached male levels after 20 days. Conversely, the growth hormone-regulated activity of transcription factor STAT5 was already detected in liver nuclei after 48 hr of androgen treatment. Furthermore, we demonstrate that activated STAT5 recognizes *in vitro* two upstream γ interferon-activated sequence (GAS) elements of the *C4-Slp* gene, centered at positions −1969 and −1831. We postulate that binding of STAT5 to these C4-Slp-specific GAS elements plays a crucial role in the chromatin remodelings that lead to transcriptional competence of the *C4-Slp* gene in the liver.**

Sex-limited protein (Slp) is an isoform of the mouse complement component C4. Both serum proteins are synthesized primarily in the liver and are encoded by two tandemly duplicated genes in the *H-2S* region (*C4* and *C4-Slp*), which exhibit striking differences in their pattern of expression. *C4* is constitutively expressed whereas, depending on the *H-2* haplotype, Slp is either undetectable in the serum or present only in sexually mature males (1). We have shown previously that the hormonal induction of *C4-Slp* in the liver is regulated indirectly by testosterone, which induces a sex-specific pattern of growth hormone (GH) secretion via hypothalamus–pituitary connections (2). *C4-Slp* can be induced by GH in hypophysectomized mice or in *Tfm* mice lacking a functional androgen receptor. In addition, male mice overexpressing GH, because of the presence of a constitutively expressed transgene, do not express *C4-Slp* (2). The rule of male-restricted expression can be violated either by pseudoalleles consisting of extra copies of *C4/C4-Slp* hybrid genes [haplotypes *H-2*^{w7}, *H-2*^{w16}, and *H-2*^{w19} (3–5)] or by trans-acting and recessive regulatory genes designated as regulators of sex limitation (*rsl*), such as those found in the FM, PL/J, and NZB strains (6). The *rsl* mutation allows not only expression in females but also increased expression in males (7). The *C4* and *C4-Slp* genes are nearly 15 kb long and have identical exon/intron organization with about 95% sequence identity in both the coding and noncoding regions (8, 9). Their transcription is driven by an initiator element (10, 11). This model allows one to study the differences between duplicated genes that result in the turning off of expression in a reversible manner by testosterone or by mutations affecting trans-acting factors. Divergent sequence elements in the 5′ flanking region of the genes have been identified, and *in vitro* studies have attempted to correlate these elements with differential expression (12–17). However, none of the slight differences in activities (2- to 5-fold) observed between the two promoters could account for the more than 100-fold difference in the steady-state levels of the nuclear mRNA precursors of C4 and C4-Slp in female hepatocytes (2, 10). An androgen-responsive *C4-Slp*-specific enhancer, located 2 kb upstream of the promoter and postulated to direct androgen-specific transcription, is involved in chromatin remodeling both in the liver and the kidney (18). Protection of this enhancer in male kidney correlates with *C4-Slp* expression. However, *in vivo* protection of the same androgen-responsive enhancer is also observed in the liver, where it is not sufficient to determine transcription of *C4-Slp* (18). The latter finding is consistent with our demonstration that expression of this gene in the liver requires a pulsatile rhythm of growth hormone secretion, not testosterone directly (2).

To resolve the inconsistencies between results obtained using various *in vitro* assays and *in vivo* chromatin studies, it was necessary to examine *in vivo* the transcription of both genes in both sexes. High levels of nucleotide identity between the *C4* and *C4-Slp* genes frustrated such *in vivo* analysis in strains expressing both genes. Mutant *H-2*^{aw18} mice result from the recessive lethal deletion of ≈80 kb of the *S* region including the active gene coding for steroid 21-hydroxylase and complement

Abbreviations: C4-Slp, sex-limited protein and isoform of the fourth component of mouse complement; GAS, γ interferon-activated sequence; GH, growth hormone.

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C4, which leaves the *C4-Slp* gene intact (19). These mice have been rescued through transgenesis with the murine *21-OHase A* gene (20) and provide the opportunity to study *C4-Slp* transcription *in vivo*, unhindered by the presence of *C4*. We therefore examined *C4-Slp* transcription initiation and elongation *in vivo* by using nuclear run-on analysis.

It has been suggested recently that the sexually dimorphic GH-dependent expression of several genes in the liver depends on the activation of the transcription factor STAT5 (21, 22). In this study, we followed the time course of appearance of STAT5 activity in liver nuclei of androgen-treated females and identified two upstream γ interferon-activated sequence (GAS) elements of the *C4-Slp* gene, which specifically bind activated STAT5.

MATERIALS AND METHODS

Animals. All animals studied were 8 weeks to 5 months old. Prepuberal males were 3 weeks old. Testosterone propionate (Sigma) was injected subcutaneously every day as a 0.1-ml volume of a 7-mg/ml solution in sesame oil for the times indicated. Control animals were injected with vehicle alone.

RNA Isolation and Analysis. Total RNA was prepared by the guanidium isothiocyanate procedure (23). Nuclear RNA was isolated by using the citric acid method, and RNase protection assay was performed as described (2). *C4* and *C4-Slp* RNA probes used in RNase protection assays were transcribed *in vitro* from inserts cloned downstream of the T3 or T7 RNA polymerase promoter in the pBS (+/−) phagemid (Stratagene) in the presence of 50 μCi of [α-^{32}P]CTP (3,000 Ci/mmol; 1 Ci = 37 GBq) as recommended by the supplier (Stratagene). The *C4-Slp*/*C4* distal probe has been described already (2). This probe discriminates C4 (100 nt) from C4-Slp (191 nt). A *C4-Slp* initiation start-site probe extending from −132 in the 5′ flanking region to +234 bp (exon 2) was obtained by PCR of genomic DNA. This region is identical in *C4* and *C4-Slp*.

Nuclear Run-On Analysis. Selection of exon-specific oligonucleotide probes for run-on analysis was based on the choice of an average length of 100 noncoding-strand nucleotides with the ability to hybridize 25 uridine nucleotides in the RNA: exon 1 probe, +21 to +119; exon 2, +222 to +320; exon 7, +1996 to +2082; exon 9, +2789 to +2886; exon 14, +4909 to +5011; and exon 33, +11934 to +12049. Complementary oligonucleotides corresponding to the coding strand were used as controls. Two additional control oligonucleotides were added: promoter region, −140 to −45; β-actin cDNA, +347 to +451. Oligonucleotides (10 μg/100 μl) were denatured at 65°C for 30 min in 0.3 M NaOH, the reaction was blocked with an equal volume of 2 M ammonium acetate, pH 7, and samples were loaded onto a nylon membrane soaked in 10× standard saline citrate (Hybond N$^+$, Amersham) by using a slot blot apparatus (Schleicher & Schuell). Oligonucleotides were fixed to the membrane by UV exposure and baking for 1 hr at 80°C.

Nuclei were prepared from pools of three livers as described (24, 25). The nuclei were resuspended in 50 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/0.1 mM EDTA/0.5 mM DTT/40% glycerol and stored at −80°C. Nuclei were incubated as described (26) with slight modifications. Briefly, 10^8 isolated nuclei were incubated in a 500-μl reaction mixture containing 25% glycerol/20 mM Tris·HCl, pH 8.0/150 mM KCl/5 mM $MgCl_2$/1 mM each of ATP, GTP, and CTP/2 mCi of [^{32}P]UTP (3,000 Ci/mmol, Amersham)/100 units of ribonuclease inhibitor (RNasin, Promega) at 26°C for either 10 min, 30 min, 1 hr, or, in pulse-chase experiments, for 10-min labeling followed by 30 min elongation with unlabeled UTP. Labeled nuclei were digested subsequently with DNase I (40 units, Amersham) for 30 min at 37°C. After addition of 100 μl of a stop mixture (200 μg/ml proteinase K/2.5% SDS/100 mM EDTA, pH 7.5) the samples were incubated for 45 min at 37°C. The RNA was extracted by using the acid phenol procedure and precipitated with isopropyl alcohol. Before hybridization, samples were partially hydrolyzed with 0.1 ml of 0.2 M NaOH for 5 min at 4°C and reprecipitated. Prehybridization, hybridization (24 hr at 65°C), and washes were carried out as described (24). For quantitation filters were exposed for 72 hr and analyzed by using PhosphorImager (Molecular Dynamics).

Nuclear Protein Extraction and Gel Mobility-Shift Assay. Nuclear proteins were extracted as described by Gorski *et al.* (27) from freshly excised individual mouse livers in the presence of classical protease inhibitors and phosphatase inhibitors: 10 mM sodium fluoride and 1 mM sodium orthovanadate. Nuclear extracts used in binding assays were finally stored in 20 mM Hepes, pH 7.9/10 mM KCl/1 mM EDTA/0.35 M NaCl/20% glycerol/1 mM DTT/1 mM phenylmethylsulfonyl fluoride/1 mM sodium orthovanadate. Double-stranded oligonucleotide probes, ^{32}P-labeled with T4 polynucleotide kinase, were incubated for 30 min at 4°C with 10 μg of nuclear extract diluted in 5 μl of storage buffer and 14 μl of gel mobility-shift buffer (10 mM Hepes, pH 8.0/50 mM KCl/50 mM NaCl/0.1 mM EDTA/5 mM $MgCl_2$/4 mM spermidine/2.5% glycerol/2 mM DTT/0.1 mg/ml BSA/2 μg of poly(dI-dC)/4 ng of double-stranded probe. In supershift analysis incubation was carried out for an additional 30 min with anti-STAT5 antibody C-17 (Santa Cruz Biotechnology) before the addition of the probe. Samples were electrophoresed at room temperature through nondenaturing polyacrylamide gels (6%) in 0.5× TBE buffer for 3 hr at 20 mA. DNA probes were the STAT5 consensus oligonucleotide AGATTTCTAGGAATTCAATC or the following oligonucleotides derived from *C4-Slp*: GCCAGTTCTCAGAACAGGCT (GAS 1, −1978 to −1960), GCTCAATTCCCAGAACCCA (GAS 2, −1841 to −1821), and AGAGTTTGAGGTCAGCCTGG (GAS 1 control), or GCTTAATTCCTAGCACCCA (GAS 2 control) from the corresponding positions of the *C4* gene in regard to the transcription start site. GTGAGGTTCCTGGAAGTGC (GAS 3, −835 to −816 in *C4-Slp*) is present in both genes.

RESULTS

***B10*^{aw18} *C4*$^{-/-}$ Mice Exhibit the Same Sexual Dimorphism of *C4-Slp* Expression as Observed in the BALB/c Strain.** *B10*^{aw18} mutant mice, which lack the *C4* gene, allow one to study *in vivo* the transcription of *C4-Slp*. Because these mutants have a large deletion within the MHC class III region, we compared the *C4-Slp* expression and the androgen inducibility in animals of this strain with those used in previous studies (BALB/c). Steady-state nuclear or total RNA isolated from the liver of both male and female *B10*^{aw18} mice were analyzed in an RNase protection assay. As shown in Fig. 1*A*, the same sexual dimorphism of *C4-Slp* expression was observed in *B10*^{aw18} mice as demonstrated previously for the BALB/c strain. Although both spliced and unspliced RNAs were detected in males (nuclear RNA, lane 1; total RNA, lane 2), neither spliced nor unprocessed RNA could be seen in female nuclear (lane 3) or total RNA (lane 4). Spliced and unspliced RNA was, however, detected in BALB/c female nuclei because of the expression of the *C4* gene (lane 6). Testosterone injection of *B10*^{aw18} females for 20 days (the time required for maximal response through the induction of the sex-specific GH secretion pattern, as shown in ref. 2) restored *C4-Slp* expression to levels comparable to those found in males (compare lanes 2 and 3 in Fig. 1*B*) or in androgen-induced BALB/c females (lane 4). Taken together, these data indicate that *B10*^{aw18} mice mimic the BALB/c phenotype in the absence of the *C4* gene and that neither the deletion comprising the *C4* gene and the *21OHase A* gene nor the *21OHase A* construct introduced through transgenesis (28) altered the hormonal regulation of *C4-Slp*.

Hormonal Control of *C4-Slp* Expression Takes Place at the Transcription Initiation Level. We then examined whether

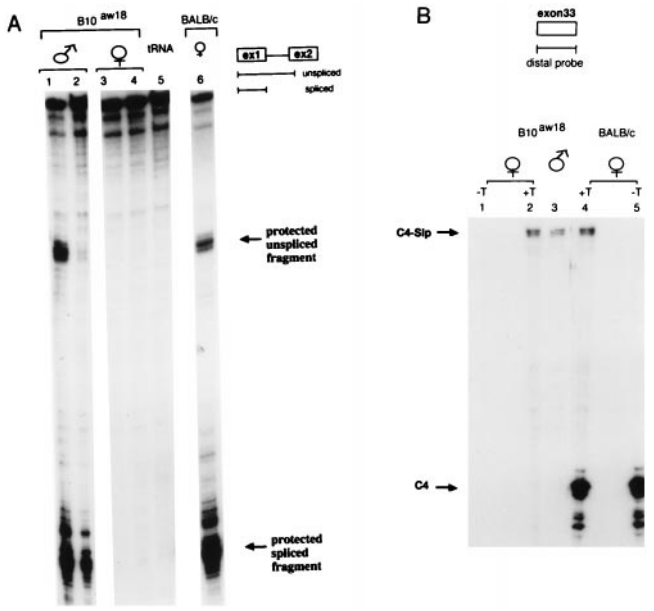

FIG. 1. RNase protection analysis of liver steady-state RNAs. (*A*) Liver RNA (30 μg) from *B10*^{aw18} mice (males, lanes 1 and 2, or females, lanes 3 and 4) or BALB/c female mice (lane 6) were hybridized to the transcription start-site probe (spliced and unspliced protected fragments of 121 nt and 234 nt, respectively, are shown schematically). Lanes: 1, 3, and 6, nuclear RNAs; 2 and 4, total RNA; 5, tRNA, negative control. (*B*) *C4-Slp* expression in *B10*^{aw18} or BALB/c females injected for 20 days with 700 μg of testosterone propionate (+T, lanes 2 and 4). A positive control is in lane 3 (*B10*^{aw18} male). Lanes 1 and 5 are untreated controls. Thirty micrograms of total RNA was hybridized to the distal *C4/C4-Slp* probe (244 nt; described in ref. 2), which distinguishes C4 (100 nt) and C4-Slp (191 nt).

lack of *C4-Slp* expression in females was a result of rapid degradation of incompletely synthesized transcripts or lack of transcription initiation. To this end, we performed run-on analyses of newly synthesized transcripts (10-min labeling) from liver nuclei of male or female *B10*^{aw18} mice, using sense and antisense oligonucleotide probes representing different regions of the *C4-Slp* gene (Fig. 2). To normalize different experiments we included as an internal control of overall transcription efficiency an antisense β-actin probe. Typical results shown in Fig. 2 reveal nascent C4-Slp transcripts in males, with an intensity comparable to that of β-actin, using a probe covering the initiation start site (exon 1). In contrast, no signal above background (e.g., antisense promoter and sense probes) was detected in females, even when using longer periods of labeling. In addition, we observed in males a strong drop in the amount of nascent transcripts detected with exon 2 and other proximal probes (exons 7, 9, and 14). This attenuation also was observed with the *C4* gene in BALB/c mice (see below). Quantitative analyses of exon 1 transcripts normalized to β-actin in several experiments are summarized in the scatter plot of Fig. 2, where individual points represent the exon1/β-actin ratios obtained by using equal numbers of nuclei from pools of three livers. Comparison of the exon 1/actin ratios indicates that a 30- to 40-fold difference between sexes is established during the transcription initiation process. (If estimated using shorter periods of labeling the difference appears higher than 50-fold.) These data demonstrate that most of the 100-fold difference seen in steady-state RNAs (2) is accounted for by differences that are already established between sexes at the transcription initiation level. We then tested whether testosterone injection for various periods of time could induce the initiation of *C4-Slp* transcription in *B10*^{aw18} females. As shown in Fig. 2, we detected similar levels of transcripts as those found in males (10 days, 20 days), using the exon 1 probe. Shorter testosterone treatment (6 days) showed incomplete induction (Fig. 2). These data are consistent with our previous findings at the level of steady-state RNA showing that the transcriptional response of females upon testosterone induction reaches male levels within ≈20 days (2).

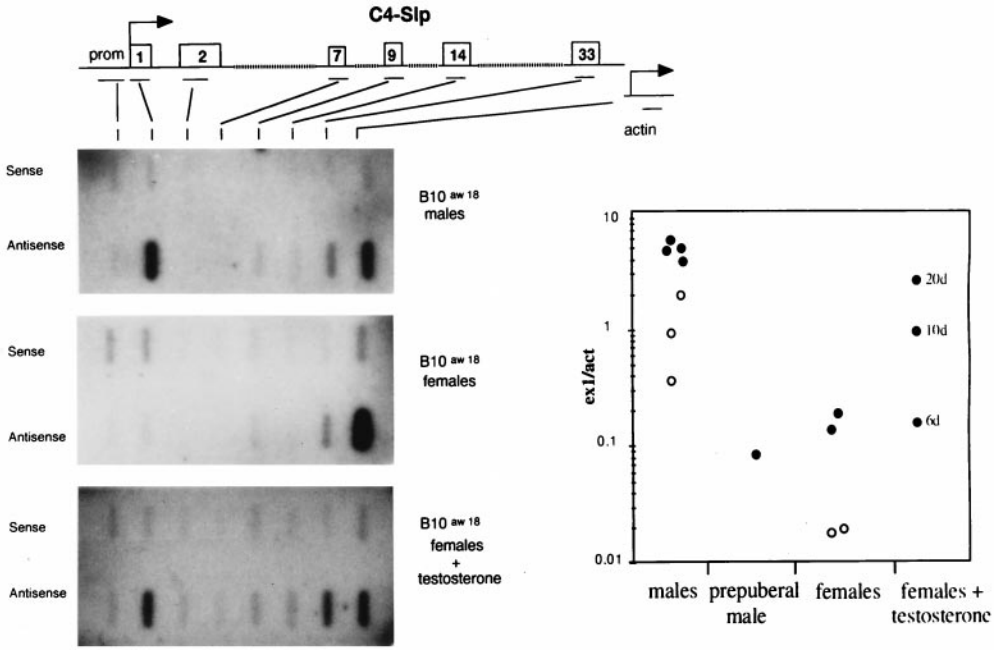

FIG. 2. Run-on analysis of newly synthesized transcripts. The sense or antisense oligonucleotides used as probes in the run-on assays are depicted on a schematic *C4-Slp* gene. Numbers represent the exons from which oligonucleotides have been designed. Two control probes (promoter and actin) are also shown schematically. The probes were immobilized on filters and hybridized with labeled nascent nuclear RNA from *B10*^{aw18} males or females, or females injected for 10 days with 700 μg of testosterone propionate. Autoradiograms are shown after 72 hr of exposure. The scatter plot summarizes different run-on experiments, each performed on a pool of nuclei from three *B10*^{aw18}livers and analyzed quantitatively by using the PhosphorImager. The ratio exon 1/act of each experiment is reported on a logarithmic scale. Long (30 min, ●) or short (10 min, ○) labeling times were used, and 6d, 10d, and 20d represent the days of testosterone treatment.

To confirm the dependence of *C4-Slp* expression on androgen induction, we tested prepuberal males for their ability to initiate transcription. It has already been shown that *C4-Slp* can only be detected in 4- to 5-week-old sexually mature males, an age at which androgen levels become significant. When run-on analysis was carried out on 3-week-old males no exon 1 transcripts were detected, a situation similar to females (Fig. 2). These data demonstrate that hormonal regulation of *C4-Slp* expression takes place at the transcription initiation level.

***C4* and *C4-Slp* Exhibit Similar Postinitiation Attenuations.** Run-on experiments in *B10*^{aw18} mice suggest a transcriptional attenuation downstream of exon 1 (Fig. 2). Control experiments demonstrated that all the different probes loaded on the filters were equally able to hybridize an *in vitro* transcribed RNA (not shown). When we verified the processivity of transcription in the isolated nuclei performing elongation for various times we found that the average size of the synthesized transcripts increased from 150 to 300 nt after 10 min of labeling (shortest time used in run-on assays) to up to 900 nt after 30 min (data not shown). To test whether this apparent attenuation was C4-Slp-specific, we performed run-on experiments in BALB/c mice. In females, which do not express *C4-Slp* but do express *C4*, and in males, which express both genes, nascent RNAs were detected with the first exon probe but a similar attenuation of the signal as exhibited by *B10*^{aw18} mice was observed with an exon 2 probe (Fig. 3). Quantitative analyses in several experiments confirmed an equivalent initiation for the two genes (ratios exon 1/actin in Figs. 2 and 3) and a strong reduction of the hybridization signal with an exon 2 probe (about 20-fold, see the exon 2/actin ratios in Fig. 3). Quantitatively similar results were obtained by using different labeling times. Moreover, when chase experiments were performed after short labeling (10 min) and further unlabeled transcription for various times, we did not observe a decrease of the exon 2/actin ratio as a function of the time of chase (data not shown). We therefore favor the hypothesis of a true attenuation of transcription (29, 30) occurring both in *C4* and in *C4-Slp* immediately downstream of exon 1, rather than that of a degradation of the nascent transcripts. The weak signals observed in Figs. 2 and 3 with antisense probes for exons 7, 9, and 14 were not detected when nuclei were incubated with α-amanitin (before and during elongation, 2 μg/ml, data not shown) and thus represent RNA polymerase II transcripts. In contrast, we observed consistently an α-amanitin-insensitive signal with a distal probe (exon 33) and RNA of different origins (males or females, *B10*^{aw18} or BALB/c strain). We therefore attribute this signal to an aberrant transcription activity not related to RNA polymerase II and not induced by testosterone.

Activation of Liver STAT5 by GH. Because the STAT5 transcription factor has been described previously to be activated by a male-specific GH secretion pattern (21, 31), we analyzed the effect of testosterone induction on STAT5 DNA-binding activity, measured *in vitro*. As shown in Fig. 4*A*, a discrete gel mobility-shift complex was formed upon incubation of an oligonucleotide containing a consensus-binding site

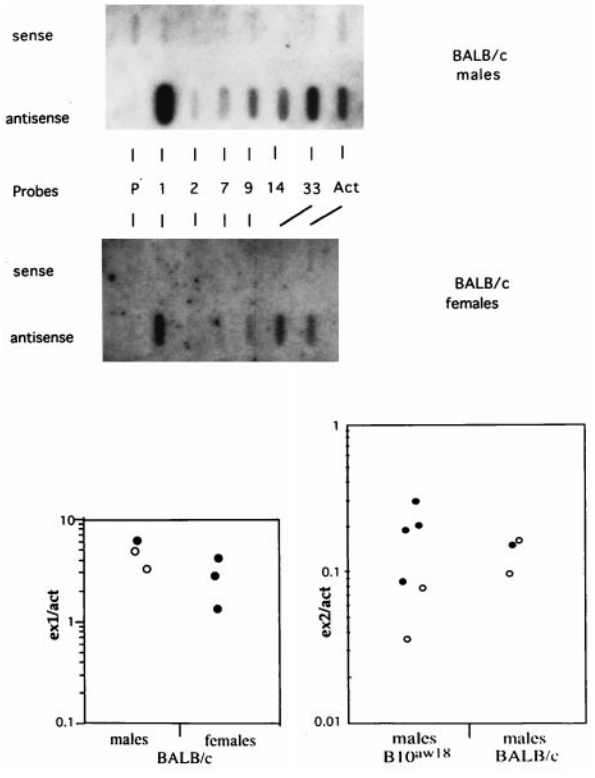

FIG. 3. Elongation run-on analysis along *C4* and *C4-Slp* genes. Probes used are identical to those described in Fig. 2. Labeled nuclear RNA was extracted from BALB/c males (30 min or 10 min) or females (30-min labeling). Autoradiograms after 72 hr of exposure are shown. The scatter plots represent exon 1/actin and exon 2/actin ratios, respectively, as obtained from several experiments performed on BALB/c males and females or on *B10*^{aw18} and BALB/c males. Long (30 min, ●) or short (10 min, ○) labeling times were used.

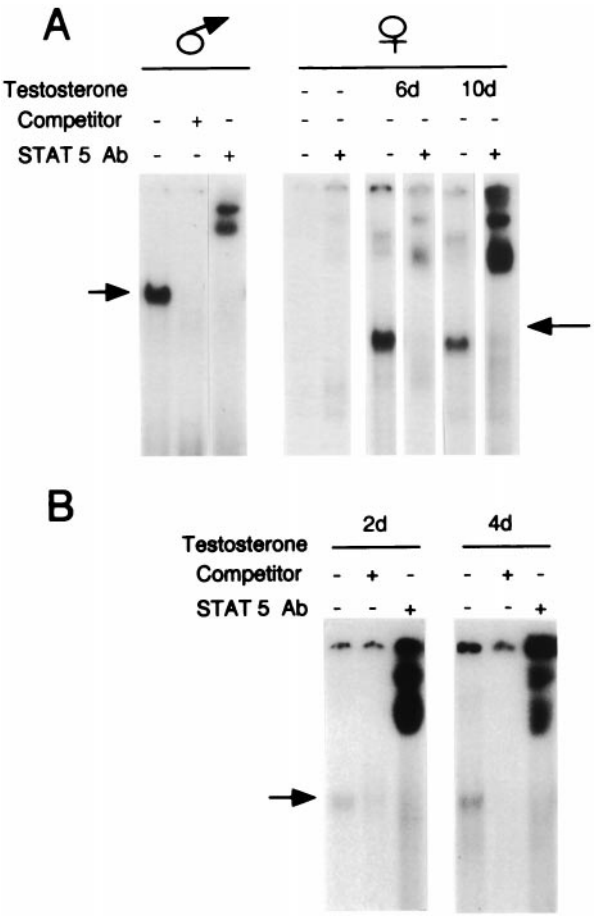

FIG. 4. Time course of liver STAT5 activation upon testosterone treatment. (*A*) Mobility shifts using liver nuclear extracts from males or from females treated with testosterone for various times. Arrows mark the position of the shifted band in experiments with different times of gel migration. The probe was an oligonucleotide carrying the STAT5 consensus sequence (see *Materials and Methods*). Exposure times were 15 hr and 48 hr for male and female extracts, respectively. (*B*) Early detection of activated STAT5 in liver nuclear extracts of androgen-treated females (2 or 4 days). The probe was the same as in *A*. An arrow points to a weak but significant shifted band observed after 2 days of androgen treatment. Exposure time was 4 days.

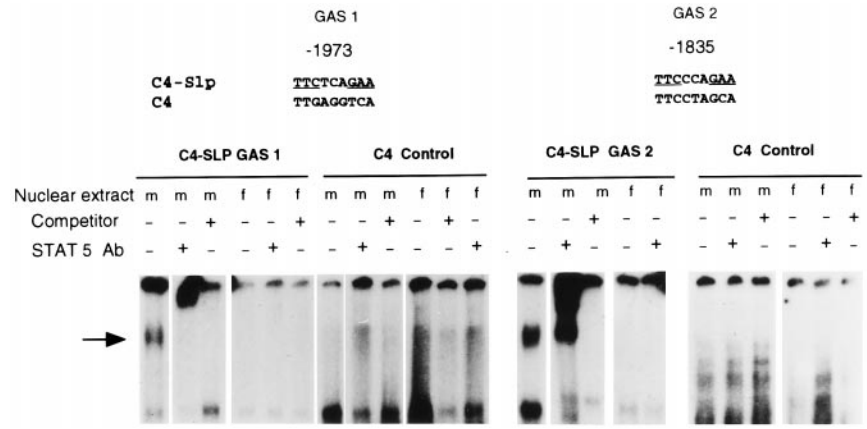

FIG. 5. Selective STAT5 binding to upstream GAS sites of *C4-Slp*. *C4-Slp* GAS sequences and the corresponding *C4* control sequences are shown along the top of the figure, and the most conserved nucleotides of the consensus sequence for STAT5 binding are underlined. Numbers indicate the position of the first T in the C4-Slp sequence. Retardation gel shift assays were performed with probes encompassing GAS 1 and GAS 2 sequences (described in *Materials and Methods*) and liver nuclear extracts from male (m) or female (f) *B10*^{aw18} mice. Competition was performed with 50-fold excess of unlabeled probe in the binding reaction. An arrow indicates the position of the protein–probe complex. Signals of various intensities at the top of each lane correspond to the origin of gel migration. Exposure was for 2 days.

for STAT5 with nuclear extracts prepared from male but not from female livers. The same complex was present in females after 6 and 10 days of androgen treatment. This complex contains STAT5, as demonstrated by the full inhibition by an unlabeled STAT5 consensus oligonucleotide and by the supershift with anti-STAT5 antibody. The detection of activated STAT5 factor already after 6 days of testosterone treatment was in contrast with the very weak transcriptional activity detected by run-on analysis (Fig. 3). Thus, we investigated STAT5 activation after 1, 2, and 4 days of testosterone injection. As shown in Fig. 4*B* a gel mobility-shift complex was already present after 2 days of androgen induction (and traces were present after 1 day, data not shown), indicating the presence of activated STAT5 in liver nuclei in spite of lack of transcription initiation.

We then investigated the binding activity of STAT5 to potential GAS sites identified in the *C4-Slp* promoter and upstream region (Fig. 5). Nuclear extracts from male and female livers were incubated with oligonucleotides corresponding to the sequences −1978/−1960 (GAS 1) and −1841/−1821 (GAS 2) in the *C4-Slp* 5′ flanking region or to the corresponding C4 sequence and to the sequence −835/−816 (GAS 3), which is identical in *C4-Slp* and in *C4*. We observed the formation of a male-specific complex after incubation with the probes containing the GAS 1 and the GAS 2 sequences of the *C4-Slp* gene. Competition with an unlabeled STAT5 consensus oligonucleotide and supershift with anti-STAT5 antibody confirmed the presence of STAT5 in the observed complex. On the contrary, the corresponding degenerated C4 sequences did not form any complex (Fig. 5). Similarly, no complex was observed after incubation with the sequence GAS3 at −835/−816 (data not shown). These findings indicate that STAT5 recognizes and binds C4-Slp-specific targets after its activation by a male-specific GH release pattern.

DISCUSSION

Sex differences in GH secretory patterns have been described as the major determinants in the sexually dimorphic expression of several liver genes. In mice, for example, the most important parameter determining male-specific gene expression is thought to be the period of low-plasma GH separating pulses, which is longer than that in females (32). Recent studies on the hamster *CYP3A-10/6β-hydroxylase* gene, which is expressed only in males and depends on a GH secretory pattern, have implicated the translocation into the nucleus of the STAT5 transcription factor upon GH-pulse-induced phosphorylation (21). This finding has been confirmed in hypophysectomized rats (31). Moreover, in STAT5b −/− mice the expression of male-specific genes like *MUP* and *CYP2D9* is abolished, suggesting that STAT5b may be the major STAT protein that mediates the sexually dimorphic effects of GH in the liver (22).

The striking differential expression of the closely related genes *C4* and *C4-Slp* (95% sequence identity) provides a unique model to study crucial sequence elements responsible for sex-specific hormonal regulation. A sex-specific pattern of growth hormone secretion induced by androgens is responsible for the 100-fold-higher levels of *C4-Slp* expression in males compared with females (2, 10). The influence of sex steroids on the pulsatile GH-secretory pattern has been investigated in rats, showing that testosterone is necessary for maintaining in adult life the long period of low GH plasma levels typical of males (33).

Here we provide *in vivo* analysis of the transcriptional regulation of *C4* and *C4-Slp* in run-on experiments performed in a context where the *C4-Slp* gene can be studied separately. We demonstrate that the sex-specific hormonal regulation of *C4-Slp* takes place primarily at the level of transcription initiation and leads to at least a 40-fold-higher transcription in males compared with females. All previous *in vitro* studies (including transfections of reporter constructs and cell-free transcriptions) aimed at comparing the promoters of these two genes failed to show more than a 2- to 5-fold difference (10). On the other hand, all attempts to express the complete *C4-Slp* gene in cell transfection have failed, in spite of the presence of a functional promoter and up to 6 kb of 5′ flanking sequences. Run-on experiments with nuclei isolated from L cells stably transfected with the entire genes moreover revealed transcription initiation only for *C4* but not for *C4-Slp* (N.V.-B., unpublished data). Oocyte injections of *C4-Slp* cosmids generated full-length transcripts of *C4-Slp*, which were aberrantly initiated 100 bp upstream of the regular liver initiation start site (our unpublished data). These results indicate that the ability to express the *C4-Slp* gene in an androgen-dependent mode depends on the presence of the natural liver-specific chromatin context, as suggested by others (18, 34, 35).

The 5′ upstream region of *C4-Slp* has been investigated hitherto under the hypothesis of androgen receptor binding to C4-Slp-specific sequences (18, 35). Prompted by recent reports on GH-dependent activation (21, 31) we demonstrated the presence of active STAT5 in male liver nuclei and in the nuclei of testosterone-treated females as early as 48 hr after the onset

of testosterone treatment (Fig. 4). We then compared the *C4-Slp* and the *C4* upstream sequences for the presence of GAS elements that could provide targets for STAT5 binding. STAT5 was found to bind specifically *in vitro* to the *C4-Slp* GAS 1 (weakly) and GAS 2 (strongly) sites but not to the sequences that are present in the *C4* promoter at the corresponding positions (Fig. 5). Interestingly, GAS 2 lies in a region of homology between *C4* and *C4-Slp*, rather than in the proviral sequence inserted upstream of *C4-Slp* (13) in which an androgen-responsive element has been described (36).

Comparison of the time course of STAT5 activation after androgen treatment with that of *C4-Slp* transcription (Figs. 2 and 4) reveals a considerable delay between signaling to the liver through the hypothalamic–pituitary axis, which requires 1–2 days, and the onset of transcription, which requires at least 6 days. A similar delay has already been observed between the very rapid (within 10 min) activation of liver STAT5 by a physiological GH pulse given to hypophysectomized rats and the slower response (1–2 days) of male-specific liver CYP gene transcription (31). These findings suggest the involvement of additional factors in the GH response. In our model, for example, the still unknown elements designated as regulators of sex limitation and determined by the *rsl* locus (7) may play a role in the chromatin remodelings (18) associated with the acquisition of transcriptional competence of *C4-Slp*.

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