Tissue-specific variation in C4 and Slp gene regulation

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ABSTRACT

C4 and Slp are highly homologous mouse genes that differ in function and regulation. Allelic variants exist in quantitative regulation of C4 and in hormonal regulation of Slp. We have examined expression in several tissues, including liver and peritoneal macrophages which are the major sites of synthesis, using a probe that allows direct comparison of C4 and Slp mRNAs. Correctly-sized and initiated RNA, within an order of magnitude of liver levels, is found in mammary gland, lung, spleen, and kidney; lower levels are detectable in testis, brain, heart and submaxillary gland. By comparing expression in congenic mouse strains differing in C4 and Slp loci, regulation of these genes is seen to vary in different tissues. This provides a well-defined genetic system in which to examine cis-acting sequences and trans-acting factors that result in tissue-specific patterns of gene regulation.

INTRODUCTION

Genes for the fourth component of complement (C4) and sex-limited protein (Slp) reside in tandem within the mouse major histocompatibility complex (H-2) (1). Extensive identity in flanking as well as coding sequences indicates that Slp has evolved from a duplicated C4 gene (2,3). Despite their similarities, Slp does not participate in the complement pathway and is stringently regulated by androgens. Characterized genetic variation in C4 and Slp expression provides a novel situation in which to correlate specific DNA sequences with various modes of gene regulation. Two major alleles of C4, C4-high (C4^h) and C4-low (C4^l), dictate 20-fold differences in serum C4 levels (1). Three alleles direct Slp expression as androgen-regulated (Slp^a), constitutive (Slp^c), or null (Slp⁰) (1,4). In addition to these cis-acting alleles (5), loci are known that affect Slp and C4 in trans (6); these may aid identification of regulatory factors.

C4 and Slp coding regions diverge less than 4% (7-9) but gene-specific probes can be obtained from regions of greater sequence variation (9,10). Some disparate regions correspond to parts of the C4 protein essential for hemolytic activity. For instance, Slp shows greatest divergence from C4 near

the site where cleavage occurs during complement activation; this is likely to account for Slp's inactivity in the lytic pathway (7,8). A similar degree of identity as in the coding regions is maintained upstream of the genes until 1.9 kb from the transcription start sites, at which point the C4/Slp homology abruptly breaks (11). It is in this 5'-flanking region of Slp-specific DNA that we have localized an androgen-responsive enhancer element (12,13).

C4 and Slp synthesis has been shown to occur in liver and peritoneal macrophages (14,15). Although in macrophages expression per cell is higher, hepatocytes are far more numerous and account for most serum C4 and Slp (as well as for most complement proteins [6]). Only macrophages from strains with constitutive Slp alleles synthesize Slp (1), which could be due to lack of androgen response in macrophages or other tissue-specific differences in C4 and Slp expression. In studying these genes, we have found that they are transcribed in more tissues than previously noted. Furthermore, comparing expression of various alleles indicates that regulation differs in different organs. This report describes expression of C4 and Slp in three congenic mouse strains differing within the H-2: B10.D2 (H-2d, C4^h, Slp^a), B10.BR (H-2^k, C4^l, Slp⁰) and B10.WR (H-2^{w7}, C4^h, Slp^c). This analysis provides a genetic framework for further molecular studies of differential tissue-specific gene regulation.

MATERIALS AND METHODS

Mice

B10.D2 and B10.BR mice, originally from the Jackson Laboratory, and B10.WR mice, from A. Ferreira and V. Nussenzweig (New York University), are maintained in Columbia's animal facility. Animals used were at least 3 months of age. For hormonal induction, female mice were injected every other day for 2 weeks with 2.5 mg testosterone propionate in 0.1 ml sesame oil.

RNA Isolation

Organs from 3 mice were pooled per point and stored at -80°C, except for brain samples, which were from single mice, and macrophages, which were prepared as follows. Peritoneal macrophages were obtained from 10 - 20 mice by washing the body cavity with Dulbecco's modified Eagle medium with 10% calf serum. Cells were allowed to adhere to plastic dishes at 37°C, 5% CO₂ for 2 hr; nonadherent cells were washed off with Dulbecco's phosphate-buffered saline. RNA was prepared from adherent cells by guanidinium-hot phenol extractions (16). RNA from all other tissues was prepared by a guanidinium-LiCl method (17). Poly(A)+ mammary RNA from Balb/c females was the gift of K. Lynch (University of Virginia School of Medicine).

Northern Blot Analysis

RNA was denatured by heating in formaldehyde-formamide, fractionated through agarose gels containing 6% formaldehyde (18), and transferred to Gene Screen Plus (NEN). Filters were hybridized to a nick-translated full-length C4 cDNA constructed from overlapping H-2^d C4 cDNA clones (9). Hybridization and washing conditions were standard.

RNase Mapping

Synthesis of single-stranded riboprobes, hybridization and RNase digestion were performed as described (19) using 20 µg RNase A and 600 U RNase T₁ per ml. To distinguish C4 from Slp RNA, a 200 bp HaeIII-HincII fragment (+2229 to +2428) of Slp^{w7} cDNA (9) was inserted into pGem4; transcription of this plasmid, pG411, from the T7 promoter results in an antisense RNA spanning a region where the Slp sequence has a 9 bp deletion and several mismatches from the C4 sequence. The plasmid used as template for synthesis of a transcription start site probe, pG407, contains an Slp^d genomic BamHI fragment (-2953 to +48) in pGem4. RNA probes were gel-purified before hybridization.

RESULTS

C4/Slp mRNA is present in several mouse tissues

In order to localize DNA regulatory elements, we previously compared the chromatin structure of C4 and Slp genes in vivo (20) and the behavior of transfected genes (13). In both studies, we noticed C4/Slp expression in cells other than hepatocytes or peritoneal macrophages. C4 and Slp genes in liver have DNase I-hypersensitive sites that correlate with their expression and with hormonal control of Slp (20). Kidney but not lung chromatin faintly showed the same nuclease-sensitive sites as liver; accordingly, low levels of C4/Slp RNA was found in kidney. In transfection experiments, a mouse mammary cell line host showed an endogenous RNA that hybridized with C4 cDNA (13).

Additional sites of C4/Slp synthesis were revealed on Northern blots of total RNA from several organs of B10.D2 adult male mice, which have high C4 and androgen-dependent Slp expression (H-2^d, C4^h, Slp^a). Northern blots were probed with C4 cDNA, which hybridizes equally well to C4 or Slp mRNA (Fig. 1). Correctly-sized 5.4 kb C4/Slp RNA was detected in all tissues tested (including mammary gland from a Balb/c mouse, which is H-2^d, C4^h, Slp^a). As levels of expression varied widely, different amounts of RNA were loaded on the gel used for Fig. 1 (see legend). Peritoneal macrophages showed the highest expression, followed by liver, as expected. Mammary gland, kidney, spleen and lung showed moderate expression, and testis, brain, heart and submaxillary

Figure 1. Northern blot analysis of C4/Slp expression in mouse tissues. RNA was electrophoresed through 0.8% agarose-6% formaldehyde, transferred to Gene Screen Plus, and probed with nick-translated C4 cDNA. A) Samples are total RNAs from B10.D2 males, except mammary which is Balb/c poly A+ RNA and Ltk⁻ which is H-2^k fibroblast total RNA. Lanes, left to right, have the following RNA amounts: macrophage - 1 μ g, liver - 4 μ g, mammary - 0.1 μ g poly A+, and 10 μ g total RNA for all others. Autoradiography was for two days. B) 10 μ g Ltk⁻ total RNA and 25 μ g total RNA from B10.WR male submaxillary gland, brain and heart were treated as in A) and the filter exposed for 6 days.

gland showed low but detectable levels of C4/Slp RNA. The only negative RNA sample was from L cell fibroblasts (from H-2^k, C4^l, Slp⁰ mouse embryo).

Alleles of C4 and Slp are differentially regulated in several tissues

To determine whether the RNA in various tissues was C4 or Slp, and whether it was regulated as in liver or macrophages, we compared expression in strains with different regulatory alleles, using a probe that distinguishes the two sequences. Quantitative regulation of C4 and hormonal control of Slp differ in hepatocytes and macrophages (10,21). Mice with C4-high alleles show 20-fold more serum C4 and correspondingly 20-fold more C4 mRNA in liver than C4-low mice (10). In contrast, peritoneal macrophages of C4^h and C4^l mice synthesize equivalent amounts of C4 protein (10), indicating that quantitative regulation of C4 is tissue-specific. Only macrophages from strains with constitutive Slp genes synthesize Slp. Both sexes of these Slp^c mice synthesize Slp in liver, whereas females with androgen-regulated Slp (Slp^a) show only 1% male levels (22). Recently it has been shown that some Slp-null strains, de-

finied by their ability to produce anti-Slp antibodies (23), actually have low levels of a slightly shorter Slp RNA in male liver (10). These allelic variants thus show tissue-specific differences in C4 and Slp expression; serum levels, upon which the nomenclature is based, reflect liver synthesis.

C4 and Slp mRNA may be distinguished with gene-specific oligonucleotides (9), but these are not very sensitive and must be used sequentially or on duplicate blots as the mRNAs are similar size. We used instead a uniformly labeled probe that identifies both mRNAs at once by RNase mapping (19). An Slp cDNA fragment (+2229 to +2428) spanning the region of greatest sequence disparity with C4 was cloned into pGem4. Transcription from the T7 promoter produces an antisense RNA that protects 200 bp of Slp mRNA from RNase (Fig. 2A). In contrast, the probe is cleaved in a region of mismatch if hybridized to C4 mRNA. Samples analyzed by RNase mapping thus show bands of 200 nt for Slp RNA and about 90 nt for C4 RNA, allowing direct comparison of mRNA levels.

Fig. 2 shows RNA of liver, macrophage and kidney analyzed in this manner from three mouse strains: B10.D2 (H-2^d, C4^h, Slp^a), B10.BR (H-2^k, C4^l, Slp⁰) and B10.WR (H-2^{w7}, C4^h, Slp^c), designated d, k, and w, respectively, in these figures. Multiple bands consistently seen for C4 may be due to inefficient digestion in the region of mismatch. 1 µg total liver RNA samples show C4 and Slp relative amounts consistent with serum protein levels. That is, Slp is abundant in d-haplotype males and both sexes of the w7 haplotype. The d and w7 haplotypes have equivalent amounts of C4 mRNA, in contrast to the k haplotype (C4^l) that has about 20-fold less. In all haplotypes, males have about twice as much C4 mRNA in liver as females, in accord with serum levels (1). When greater amounts of RNA are analyzed, d-haplotype females show a tiny fraction of the Slp RNA of males, consistent with radioimmunoassays of serum protein (22). Both sexes of the k haplotype also contain Slp mRNA (but not immunoprecipitable protein [22]) in a 20-fold reduced but analogous fashion to the d-haplotype, i.e. androgen-regulated. Thus at the RNA level this Slp⁰ allele is low rather than null, like the C4 gene in this strain.

Macrophage RNA levels agree with expression characterized by protein synthesis (21). In Fig. 2C, 2 µg total peritoneal macrophage RNAs are shown compared to 1 µg of d-male liver RNA (used for comparison on subsequent gels as well). C4 synthesis in macrophages is several-fold greater than in hepatocytes and is equivalent in these cells regardless of haplotype. Only animals with constitutive Slp alleles show Slp mRNA. Minor male/female differences were not reproducible and may reflect poorer quantitation of small amounts of RNA or differential contamination with other cell types.

Figure 3. Androgen-dependence of Slp expression in liver and kidney. B10.D2 male mice were castrated (-T) and sacrificed at 6 wks or 3 mos; B10.D2 females were treated with testosterone (+T) and sacrificed at 3 days or 2 wks. RNAs from liver (1 μ g) and kidney (20 μ g) were analyzed with the C4/Slp probe as in Fig. 2. Panels are from the same autoradiogram. M: HpaII-cleaved pBR322 DNA.

The level of C4 RNA in d kidney is about 1/10th that of liver and higher than in the other haplotypes (comparing 20 μ g kidney RNAs to 1 μ g d-male liver RNA; Fig. 2D). Slp mRNA is also higher in the d haplotype and higher in males than females for all strains. The ratio of Slp to C4 mRNA is greater in kidney than in liver. Thus regulatory features beyond quantitative control of C4 and Slp apparently differ in kidney. That C4 RNA is higher in the d haplotype, while k and w7 haplotypes are roughly equivalent, is like neither the equivalence of expression in macrophages nor the marked k haplotype depression in liver. More strikingly, Slp is expressed at high levels and in a sex-regulated manner regardless of haplotype in kidney.

Androgen-dependence of Slp expression was compared in kidney and liver of hormonally manipulated B10.D2 mice (Fig. 3). Slp mRNA declines slowly in male liver following castration, with substantial RNA still present after 3 months. In kidney, the decrease is faster; after 3 months Slp is barely detectable. Androgen induction of Slp expression in females occurs more rapidly in kidney

Figure 4. Expression of C4 and Slp in lung, spleen and submaxillary gland. Total RNAs were analyzed as in Fig. 2 with the C4/Slp-specific probe. Notation is as in previous figures. A) 20 μ g lung RNAs are shown in comparison to 1 μ g d σ liver RNA (L) analyzed on the same gel. P, probe; M, HpaII-digested pBR322. B) 20 μ g spleen RNAs are shown as in A, compared to 1 μ g d σ liver RNA (L) from the same autoradiogram. C) 20 μ g submaxillary gland total RNA was analyzed as in A, with 1 μ g d σ liver RNA (L) from the same gel.

than in liver (not shown). Differences in rate or extent of androgen action may be due to involvement of other regulatory factors.

The lung, spleen and submaxillary gland qualitatively regulate Slp and C4 similarly to each other (Fig. 4). Lung and spleen express about 1/10th and submaxillary gland 1/50th the liver C4 levels (comparing 1 μ g liver RNA to 20 μ g RNA from the other tissues). The quantitative allelic variation in liver C4 synthesis is seen also in these organs, though not as markedly. Doubling of C4 levels in mature males does not occur in these tissues as in liver. Significant Slp RNA levels are only detected in the w7 haplotype, in both sexes, as in peritoneal macrophages.

Heart and brain express very low levels of C4 (Fig. 5), similar to submaxillary levels; however, the difference between high and low C4 alleles is not pronounced. In heart, Slp is detected in w males and to a lesser extent in w females; in brain, Slp is detectable in all haplotypes and both sexes.

Figure 5. Expression of C4 and Slp in heart, brain, testis and mammary gland. RNAs were analyzed as in Fig. 2; notation is as before. A) 20 μ g heart total RNAs are shown with 1 μ g d σ liver RNA (L) analyzed alongside. B) 20 μ g total brain RNAs are shown with 1 μ g d σ liver RNA. The d σ brain sample was overdigested (indicated by fainter residual probe and Slp signal relative to other samples). d o samples of both heart and brain have anomalously high C4 signals, although actin levels on Northern blots indicate equivalence of all samples. C) 20 μ g testis total RNA from d, k and w σ s and 0.4 μ g poly A+ mammary gland RNA of Balb/c females is compared to 1 μ g d σ and ϕ liver RNAs.

Testis shows higher C4 and Slp expression than brain or heart and relatively high Slp/C4 ratios in the w7 haplotype (Fig. 5C). Only d haplotype mammary gland was examined; C4 expression was quite high while Slp was not detected. The prominent liver transcription start site is used in most tissues

To examine whether transcripts of C4 and Slp in different organs derive from tissue-specific promoters, we mapped the 5' ends of the mRNAs. A ribo-probe used to map liver start sites protects the first 48 nt of both C4 and Slp mRNAs; there are two base differences between C4 and Slp in the untranslated region but neither is cleaved in this assay (Fig. 6A). The site designated +1 accounts for the vast majority of transcripts in liver and has been defined as well by primer extension (9) and S1 analysis (24).

Fig. 6B shows RNase mapping with the antisense probe extending from +48 to -95 of the Slp sequence. In most tissues, regardless of haplotype, the

Figure 6. Transcription start sites of C4/Slp RNAs in different tissues and haplotypes. A) Probe for 5' end-mapping is from a pGem plasmid containing an Slp genomic fragment extending from +48 to -2953 relative to the liver cap site. The plasmid was linearized with BglIII at -95 and transcribed with T7 polymerase; 48 nt of the 182 nt riboprobe is complementary to both C4 and Slp mRNA 5' ends. Transcripts initiating upstream of -95 protect 145 nt of probe (37 nt is derived from the plasmid). B) RNAs were hybridized and digested with RNase as above. Samples are from males unless noted (d o liver, kidney and mammary). Lanes: M, HpaII-cleaved pBR322; P, probe alone; L, liver RNAs; K, kidney; Mø, macrophage; T, testis; M, mammary gland; S, spleen; Lu, lung; H, heart; B, brain; Sm, submaxillary gland; F, frog poly(A)- RNA. Liver lanes, left to right: 20 μ g in the first two lanes (d o and k o); 2 μ g in the next three lanes (d o, d q and w o). All other samples are 20 μ g total RNA, except 1 μ g macrophage and 0.4 μ g poly(A)+ mammary RNA. Open arrow, undigested probe; thin black arrow, protected probe minus plasmid sequences; thick arrow, the characterized liver transcription start site.

same start site used in liver accounts for the majority of transcripts. In some cases, however, the probe (minus transcribed polylinker sequences) is fully protected by the tissue RNA, indicative of transcripts deriving from farther upstream. These are particularly striking in testis and brain; in accord with their pattern of constitutive Slp expression, this may indicate a lack of regulation that exists in other tissues. In the k haplotype, both spleen and lung also show some transcripts initiating beyond -95 nt. Mapping experiments with a longer probe indicate that in testis, at least, upstream transcripts initiate at about -300 nt (not shown).

DISCUSSION

This report describes C4 and Slp gene expression in cells other than hepatocytes or peritoneal macrophages, which are the major characterized sites of complement biosynthesis. While some sites of expression (testis, brain, heart, submaxillary gland) show very low amounts of mRNA, other organs (kidney, spleen, lung, mammary gland) have levels within an order of magnitude of liver expression. By comparing expression among mice with various C4 and Slp alleles, it is apparent that these genes show strikingly different tissue-specific as well as strain-specific regulation. Modes of expression are categorized in Table I; additional regulatory classes may exist within these broad groupings. The differences in regulation are not due primarily to use of alternative promoters as additional transcription start sites are apparent only in brain and testis.

A major question arising from this study concerns the cellular origin of C4/Slp expression. Resident macrophages occur in all tissues and exhibit regional differences in their morphology, biochemistry and function (25). Lung and spleen macrophages synthesize C4 (26), but resident liver macrophages, Kupfer cells, do not (14). If resident macrophages account for C4 and Slp synthesis in some tissues, they show remarkably different gene regulation, due either to intrinsic differences in macrophage subpopulations or environmental factors (27). For instance, lung, spleen and submaxillary gland show quantitative haplotype-dependence of C4 synthesis (high or low), like liver, while peritoneal macrophages express similar C4 amounts regardless of haplotype. In contrast to C4 regulation, Slp regulation in these tissues is similar to macrophages in that it is only apparent in Slp^C strains.

Circulating monocytes can synthesize all the complement components (28). Thus the low level of C4/Slp expression in heart, which is qualitatively similar to that of peritoneal macrophages, could be due to residual blood cells.

TABLE I. Summary of C4 and Slp expression in various tissues.

ORGAN	Level ^a	C4 regulation ^b	Slp regulation ^c
Liver	(1)	Q	A
Peritoneal macrophage	5	E	W
Kidney	0.1	K	A
Lung	0.1	Q	W
Spleen	0.1	Q	W
Submaxillary gland	0.02	Q	W
Heart	0.02	E	W
Brain	0.02	E	C
Testis	0.05	E	(A or C)
Mammary gland	0.2	n.d.	(A or W)

^aApproximate level of expression of C4/Slp in tissues of B10.D2 male mice (except for Balb/c mammary gland) relative to expression in liver was assessed by densitometric scanning of Northern blots.

^bC4 regulation is described as quantitative (Q) if high-low allelic differences are seen as in liver, equivalent (E) if levels are similar regardless of haplotype as in peritoneal macrophages, or kidney-specific (K) to indicate higher expression of C4 in the d haplotype.

^cSlp regulation is designated androgen-regulated (A) if significantly higher in males than females, w7-specific (W) if significant only in both w7 sexes, and constitutive (C) if present similarly in both sexes of all haplotypes. n.d. Not done for all haplotypes.

Some cells other than monocyte-macrophages or hepatocytes have been shown directly to synthesize complement components, such as astroglial cells (29).

While kidney macrophages are likely to express C4/Slp, as they synthesize C2, C3 and factor B (30), indirect evidence suggests that other cell types in kidney contribute to C4 and Slp expression. DNase I hypersensitivity of C4 and Slp genes in kidney is similar to that in liver, but is not detected in lung (20). This may indicate that a small subset of lung cells are responsible for C4/Slp synthesis whereas a larger number of cells express in kidney, such that an active chromatin configuration is detected. As level of expression in the two organs is similar, expression in a subpopulation (i.e., macrophages) in lung would be greater per cell than in kidney. Kidney expression may reveal more about the androgen regulation of Slp, which is seen in kidney regardless of haplotype. The hormonal regulation in kidney may differ from that in liver, where growth hormone is involved in androgen effects (31).

The Slp-null allele of B10.BR mice expresses like an androgen-regulated low allele. That is, in all tissues expressing Slp^a, Slp⁰ mRNA is present; in tissues where C4 is quantitatively regulated, Slp⁰ is low, but in tissues where the C4 quantitative difference is not seen, Slp⁰ expresses on a par with Slp^a. Tissue-specific low expression of C4 and Slp in this strain could be

due to a general effect on the locus, or to a mutation in one gene that was secondarily acquired by the other by recombination, as seen elsewhere in this system (9,12,32). The liver Slp⁰ RNA is somewhat shorter than other Slp mRNAs (10); a deletion could result in a truncated product by introduction of a termination codon or an altered protein that is not antigenically demonstrable.

Slp expression in B10.WR mice is also more complex than initial analyses have shown. Strains with H-2-linked testosterone-independent Slp expression possess multiple Slp genes; apparently, recombination with the single C4 gene in 5'-flanking regions accounts for their C4-like (constitutive) regulation (12,32). We have found that one of four B10.WR Slp genes is more similar to the Slp^a gene in its 5' flank, and, like that allele, an upstream region can confer androgen-responsiveness on a reporter gene in transfection assays (12). That this Slp gene expresses in vivo has not been shown; cDNAs isolated from the w7 haplotype represent two of the constitutive-type genes (24). Thus it is intriguing that Slp expression in the w7 haplotype in kidney is androgen-regulated. Determining which Slp genes in this mouse express in kidney should be informative for hormonal as well as tissue-specific regulation.

With a sensitive probe allowing simultaneous comparison of C4 and Slp sequences, we have examined expression of these genes in a variety of tissues. Because of the rich genetics of the system, simple strain comparisons reveal the differential regulation of these genes in different tissues. Defining the cell types accounting for expression will indicate the relative influence of genetic and epigenetic factors in regulation. The basis of this regulation may then be elucidated by molecular approaches using differentiated cell lines and cloned C4 and Slp genes.

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