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Multiple C4/Slp Genes Distinguished by Expression after Transfection

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The *S* region of the murine major histocompatibility complex contains two closely related genes: C4, encoding the fourth component of complement, and Slp, encoding sex-limited protein. We cloned these genes from a cosmid library of the B10.W7R strain that does not show androgen regulation of the Slp protein. Restriction site polymorphisms revealed at least four C4-like genes within the S^{w7} locus, indicating evolutionary amplification of this region. Transfection of these genes into L cells resulted in expression, processing, and secretion of immunologically correct C4 and Slp proteins. At least two different Slp genes and one C4 gene were capable, after transfection, of expressing C4 and Slp indistinguishable from macrophage-derived protein. A third Slp gene exists within this locus whose recombinant cognate did not express in L cells. Thus, the B10.W7R *S* region includes one C4 gene and at least three Slp-like genes.

The *S* region of the mouse major histocompatibility complex (*H-2*) includes the genes for two very closely related proteins, C4 (the fourth component of complement) and Slp (sex-limited protein), that are distinct in their structure, function, and regulation (23). This system is remarkable owing to the extensive homology of the two genes and the variety of regulatory alleles existing within the *S* locus in congenic strains of mice. Both C4 and Slp are synthesized in liver and macrophages as 200,000-molecular-weight single-chain precursors that are similarly processed to three-chain disulfide-linked serum glycoproteins (11, 22). Despite extensive structural similarities of C4 and Slp, including partial amino acid homologies (13), Slp has no hemolytic activity (9) and is regulated by testosterone (19). In humans, there are two C4 genes within the major histocompatibility complex which are both functional in the complement pathway (1), in contrast to the divergence and loss of C4 function for the duplicated mouse gene.

Several alleles exist for both C4 and Slp that represent regulatory as well as structural differences in the proteins. Serum concentrations of C4 vary 20-fold as dictated by two major alleles, C4-high ($C4^h$) and C4-low ($C4^l$) (23). Three alleles have been described which affect regulation of Slp: (i) testosterone-regulated expression (Slp^a), (ii) constitutive expression (Slp^{w7}), and (iii) null expression (Slp^0) (12, 20). The Slp^{w7} protein has been shown to have at least some structural variation from the Slp^a protein, in addition to its regulatory difference (8). The congenic strain (B10.W7R) showing constitutive Slp expression has a $C4^h$ allele that also shows structural variation as indicated by the reduced hemolytic efficiency of this C4 molecule (2).

To study the structure, regulation, and evolution of C4 and Slp, several groups have obtained cDNAs specifying portions of the human and mouse C4-like genes (3, 17, 18; Hemenway et al., submitted for publication). Chaplin et al. (5) have isolated a cluster of cosmid genomic clones spanning 240 kilobases (kb) of the *S* region of the BALB/c mouse ($H\text{-}2^d$). Two C4-related cosmid clones designated C4-X and C4-Y that map about 50 kb apart were transfected into mouse L-cell fibroblasts to determine whether these clones could express correct C4 or Slp proteins (4). One of the cosmids, encompassing the C4-Y gene, encoded a hemolytically active C4 protein. The other cosmid, presumably Slp, did not express either at the RNA or at the protein level, probably owing to an incomplete 5′ end that lacks sequences necessary for transcription.

In this report, we describe cosmid genomic clones isolated from the B10.W7R strain that is constitutive for Slp expression. Both C4 and Slp proteins can be expressed from these clones in L cells, allowing us to distinguish intact C4 and Slp genes and to begin to define the boundaries of these sequences. Ultimately, we wish to compare regulatory features of different Slp alleles. This is particularly interesting for the B10.W7R strain in that this *S* locus contains multiple C4/Slp genes. Our data indicate the existence of at least three different (nonallelic) Slp-like genes, but a single C4 gene, in this congenic strain.

MATERIALS AND METHODS

Materials. Restriction and modification enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The construction and screening of the cosmid library and preparation of cosmid DNAs were all as described by Steinmetz et al. (24). Dulbecco modified Eagle medium was from Flow Laboratories, Inc. (McLean, Va.), and calf serum was from M.A. Bioproducts. [^{35}S]methionine (>1,000 Ci/mmol) was from Amersham Corp. (Arlington Heights, Ill.). ^{32}P-nucleotides, En^{3}Hance, and Gene Screen Plus were from New England Nuclear Corp. (Boston, Mass.). Protein A-Sepharose was from Pharmacia (Uppsala, Sweden). Ss and Slp antisera were the gift of V. Nussenzweig (9).

Cell culture and DNA-mediated gene transfer. Mouse tk^- L

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cells were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum. Cells were transformed with 1 ng of ptk (the 3.6-kb *Bam*HI fragment containing the HSV *tk* gene inserted into pBR322), 1 to 5 μg of cosmid DNA, and 15 μg of carrier Ltk$^-$ DNA per 10^6 cells, as described by Wigler et al. (25). Transformants were selected and maintained in Dulbecco modified Eagle medium with 10% calf serum, 15 μg of hypoxanthine per ml, 1 μg of aminopterin per ml, and 5 μg of thymidine per ml (HAT medium).

Southern and Northern blotting. High-molecular-weight DNA and total cellular RNA were prepared as previously described (6, 21, 25). DNA was digested to completion with 2 U of restriction enzyme per μg of DNA, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose. RNA was denatured by heating in formaldehyde-formamide, fractionated through agarose gels containing 6.0% formaldehyde (15), and transferred to Gene Screen Plus. Filters were hybridized to ^{32}P probes labeled by nick translation of C4 and Slp cDNAs isolated in this laboratory (Hemenway et al., in preparation). In some experiments, C4- and Slp-specific single-stranded oligonucleotide probes were synthesized on an Applied Biosystems model 380A DNA synthesizer and ^{32}P end labeled with T4 polynucleotide kinase. Hybridization and washing conditions were standard.

Nucleic acid probes. cDNA probes used in this study derive from C4 and Slp cDNA clones of both *H-2*^{d} and *H-2*^{w7} mice. Sequence comparisons reported (17, 18) and unpublished data from this laboratory indicate up to 95% homology for most regions of C4 and Slp, and thus long cDNA probes hybridize equally well to both sequences. The cDNA probe used in Fig. 1 was a 1.2-kb C4 fragment from the 3′ end of the mRNA which simplifies the blot pattern otherwise obtained for these large genes. This cDNA fragment encompassed the coding region for the last 160 amino acids of the α peptide chain and all but the last 30 amino acids of the γ chain. cDNA probes used in other experiments (Fig. 3 and 6) encompassed about 3.6 kb of the C4 mRNA, extending from the poly(A)-addition site. C4- and Slp-specific probes were synthesized to regions where the C4 and Slp sequences diverge significantly. One region occurs immediately 3′ to the COOH terminal of the C4a peptide, as also noted by Nonaka et al. (17). Single-stranded oligonucleotides (23 bases) synthesized to this region of the C4 and Slp sequences differ in 12 of the 23 nucleotides. A second region of marked sequence divergence, as also noted by Ogata and Sepich (18), occurs shortly before the cleavage signal between the α and γ peptide chains where C4 and Slp differ in 7 of 25 bases. Oligonucleotide probes synthesized to this region also hybridized specifically to C4 or Slp.

Biosynthetic labeling and immunoprecipitation of C4 and Slp. Cells (2×10^6) were plated in 60-mm culture dishes and incubated overnight in HAT medium plus 10% calf serum. Cells were starved for 20 min in methionine-free Dulbecco modified Eagle medium with 10% dialyzed fetal calf serum and then radiolabeled for 2 to 4 h in 1 ml of fresh medium with 100 μCi of [^{35}S]methionine. After incubation, the medium was harvested, and cells washed thoroughly and lysed as previously described (10). Peritoneal macrophages from two to four mice per dish were treated essentially identically. Samples of lysates and media were incubated at 0°C for 1 h with an excess of anti-Ss or anti-Slp serum. Immune complexes were precipitated with protein A-Sepharose, washed, solubilized, reduced, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14). Fixed gels were treated with En^{3}Hance, dried, and fluorographed. Radioimmunoassays from dishes of confluent cells were performed as described in detail previously (7).

RESULTS

Multiple C4/Slp genes occur in the *H-2*^{w7} *S* locus. The C4/Slp genes of several congenic mouse strains were initially examined to correlate differences in gene structure with different modes of C4/Slp expression. C4 and Slp cDNA sequences are about 95% homologous for regions examined so far and thus cross-hybridize extensively (see above), yet expression of the genes is markedly dissimilar. The B10.W7R strain (*H-2*^{w7} haplotype) shows high expression of C4 (C4^h) and constitutive expression of Slp (Slp^{w7}); B10.D2 mice (*H-2*^{d}) show high expression of C4 and testosterone-regulated expression of Slp (Slp^a) (23). Neither B10.BR (*H-2*^{k}) nor B6/KH (*H-2*^{b}) strains express Slp (Slp^0); the former strain exhibits low expression of C4 (C4^l), while the latter is C4^h (23). Restriction site polymorphisms have been found that distinguish each haplotype.

A different blot pattern was obtained for each haplotype when a 1.2-kb C4 probe from the 3′ portion of the cDNA was hybridized to *Hin*dIII-digested genomic DNA (Fig. 1). Molecular maps of the *S* locus from an *H-2*^{d} mouse (5) indicate that the Slp^a gene is largely encompassed within the 23-kb *Hin*dIII fragment (18). The C4 gene has internal *Hin*dIII sites resulting in hybridization of the 3′ cDNA probe to 4.8- and 6-kb C4 fragments. The C4 4.8-kb *Hin*dIII fragment is the only band present in all four haplotypes. In the *H-2*^{k} haplotype, sequence polymorphisms may correlate with low C4 expression. Neither *H-2*^{b} nor *H-2*^{k} mice express Slp; the 23-kb *Hin*dIII fragment characteristic of Slp^a is present in the *H-2*^{k} haplotype but not in the *H-2*^{b}, indicating possible allelic variants of the Slp^0 phenotype.

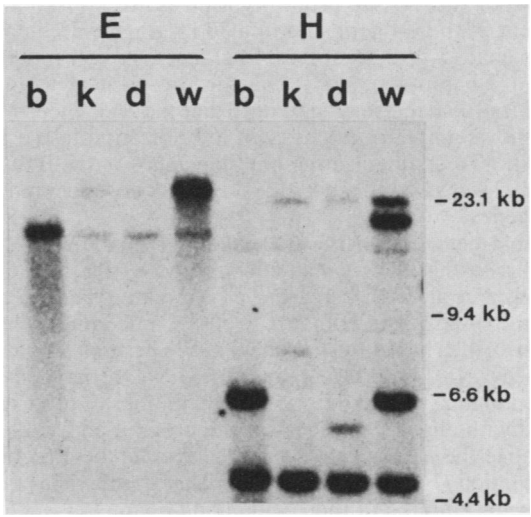

FIG. 1. Hybridization of mouse liver DNAs with C4 cDNA. DNA (15 μg per lane) was digested with *Eco*RI (E) or *Hin*dIII (H), electrophoresed on a 0.8% agarose gel, and Southern blotted as described in the text, and the filter was hybridized to a 1.2-kb C4 cDNA probe from the 3′ portion of the coding sequence. This probe encompasses the coding region for the last 160 amino acids of the α peptide chain and all but the last 30 amino acids of the γ chain. Haplotypes indicated above each lane are those of the following strains: B6.KH (b); B10.BR (k), B10.D2 (d); B10.W7R (*w7*; indicated as lane w). Molecular weight markers were from a *Hin*dIII digest of λ DNA.

TABLE 1. Expression of B10.W7R cosmids in transfected L cells

Cell line	Cosmid	Oligo[a]	5.7-kb mRNA[b]	Slp RIA (cpm)[c]
1	4.4	C4	−	ND[d]
2	8.1	C4	−	ND
3	9.1	Slp	+	87
4	11.1	Slp	−	0
5	13.4	Slp	−	ND
6	15.3	Slp	+	1,332
7	16.2	C4	+	25
8	27.2	Slp	+	5,280
9	35.6	C4	+	ND
10	38.3	Slp	+	1,800
11	43.7	C4	+	99
12	49.3	C4	+	14
13	59.1	Slp	+	ND
14	60.2	Slp	+	314
15	12.5	C4	+	ND

[a] Specific oligonucleotide hybridizing to cosmid clone DNA. The 23-mers made to the C4 and Slp cDNA sequences near the COOH terminus of the C4a peptide are only 50% homologous to each other in sequence, unlike other portions of the cDNAs which show 95% homology (17), and are thus C4- or Slp-specific.

[b] Expression of 5.7-kb mRNA shown to cross-hybridize with C4/Slp cDNA on Northern blots.

[c] Counts per minute above background in RIA of cell media. Ltk$^-$ cells (background) showed 473 cpm in this assay; B10.W7R sera diluted 1/2,000 showed 8,345 cpm above background.

[d] ND, Not done.

The *H-2*^{w7} hybridization pattern is the most complex, showing a greater number and intensity of hybridizing fragments. As also noted by Levi-Strauss et al. (16), the testosterone-independent expression of Slp in the *w7* haplotype seems to correlate with multiple C4/Slp genes, whereas two C4-like genes are present in other haplotypes. This can be seen most clearly in the *Eco*RI digestion of Fig. 1. In the *d* haplotype, the cDNA probe hybridizes to a 17-kb *Eco*RI fragment of the C4 gene and to a 30-kb fragment containing the Slp gene (5, 18). This 30-kb fragment is greatly intensified in the *w7* haplotype, indicating the presence of additional genes. It is also interesting to note that the *H-2*^{b} mouse (C4^h, Slp^0) shows a greater intensity of hybridization to the 17-kb fragment. To distinguish the multiple genes of the B10.W7R *S* locus, we isolated and characterized cosmid clones of these genes.

Cosmid clones from the B10.W7R *S* region. A B10.W7R genomic cosmid library was constructed by the methods of Steinmetz et al. (24), using the pTL5 vector, and screened with a 300-base-pair human C4 cDNA encoding a central portion of the human C4 α chain (3). The first four clones obtained (2.1, 41.1, 41.2, and 41.3) were mapped with restriction enzymes to determine possibilities of overlap and probed with oligonucleotides specific to C4 α and γ subunits to localize the coding regions and determine the direction of transcription (Fig. 2). These cosmids showed similar coding regions but differed in their 3′ flanking regions, as indicated perhaps best by differences in *Sma*I and *Bam*HI sites. Thus, these clones seem to represent three different genes, as seen by the restriction site differences.

The cosmid library was rescreened with probes that encompassed more coding information (the *Sma* E and G fragments [Fig. 2]), and 47 strongly hybridizing colonies were picked. An additional polymorphism within these clones was detected by hybridizing *Sma*I-digested DNAs with the *Sma*-G probe after Southern transfer. For most of the cosmids (including those of Fig. 2), a 0.9-kb *Sma*I

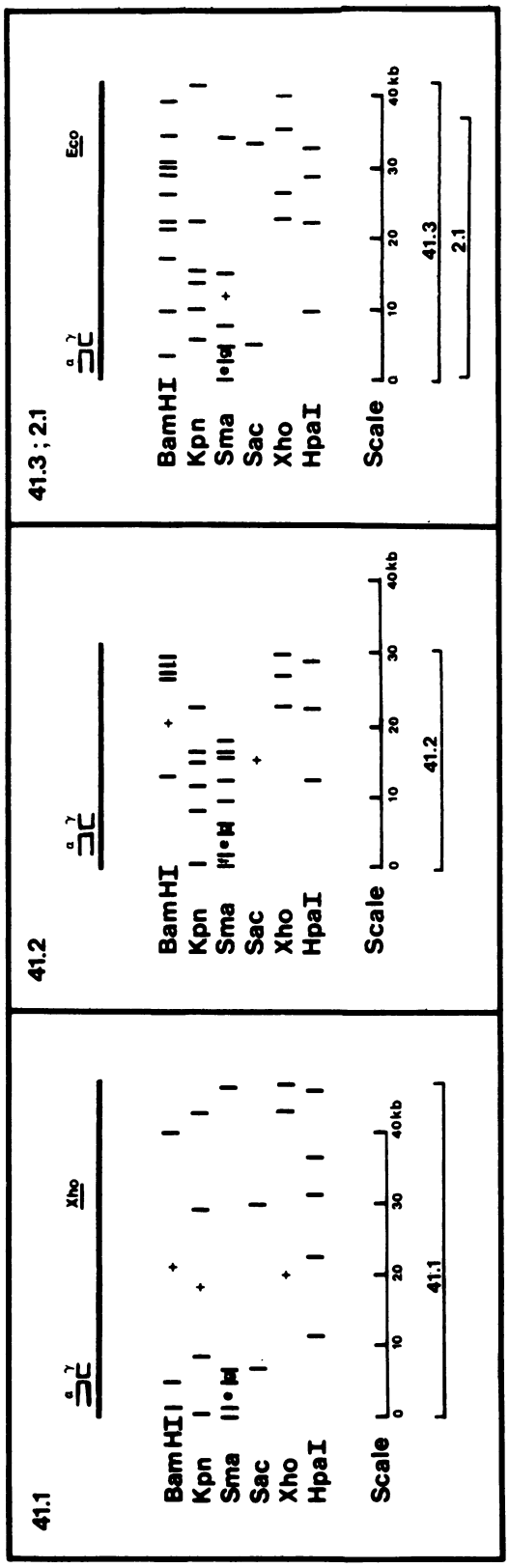

FIG. 2. Restriction maps for three partial *S* locus genes derived from four cosmid clones. Cosmid clones were mapped for the indicated enzymes by single and double digestions. DNA was electrophoresed on agarose gels which were stained with ethidium bromide and photographed, and DNA fragments were transferred to nitrocellulose filters. These filters were hybridized with the human C4 cDNA probe (3) and a γ-chain-specific probe to identify those respective coding regions (open boxes) and thus define the orientation of transcription, which is left to right, 5′ to 3′, for all these clones. A + between two restriction sites indicates the presence of additional recognition sites for the enzymes that were not mapped. *Eco*RI and *Hin*dIII sites were not completely mapped but are numerous in flanking regions. *Sal*I and *Cla*I do not cut within any of the inserts. Cosmid 41.3 seems to overlap entirely cosmid 2.1. The *Sma*I F, E, and G coding-region fragments are shown within the *Sma*I restriction sites; the *Xho* and *Eco*RI flanking-region probe fragments are shown above the cosmid line. The Slp-specific oligonucleotide probe synthesized to a sequence just before the α-γ junction (see Materials and Methods) hybridizes to all these clones, identifying them as Slp genes.

fragment hybridized to the *Sma*-G probe, whereas other cosmids showed a 3.7-kb fragment hybridizing instead. Cosmids with the 3.7-kb fragment represent a gene in addition to the first three characterized. Thus, restriction site polymorphisms within the cosmid clones indicate that there are at least four different C4-like genes within the B10.W7R *S* region.

Transfection and expression of C4-like genes. To identify which cosmids encode intact C4-like genes and whether these are capable of synthesizing immunospecific C4 or Slp protein, we introduced several cosmids into mouse L cells. Since data from this laboratory and others (5) indicate that the C4 gene is about 20 kb in length, we chose for transfer those cosmids which hybridized to coding-region probes, but not to 3′ flanking probes (Fig. 2). Therefore, these clones could contain no more than 20 kb 3′ to the gene and were likely to have sufficient regulatory and coding information to specify transcription of C4 or Slp. Of the 51 cosmids, 15 had this hybridization pattern (Table 1). These 15 cosmids were further identified by hybridization to oligonucleotide probes specific for C4 or Slp that were derived from cDNA sequence differences at the COOH terminus of the C4a peptide (see above). Of these 15 cosmids, 7 hybridized to the C4 probe, and 8 hybridized to the Slp oligomer.

These 15 cosmid DNAs were individually cotransfected into Ltk^- cells by using ptk as the selectable marker (25). For each of the 15 cotransfections, 9 independent TK^+ colonies were picked and grown up as stable cell clones. In Southern blots of total DNA from TK^+ cell clones, donor sequences were identified by hybridization with C4 and Slp cDNA probes (data not shown). Transferred genes capable

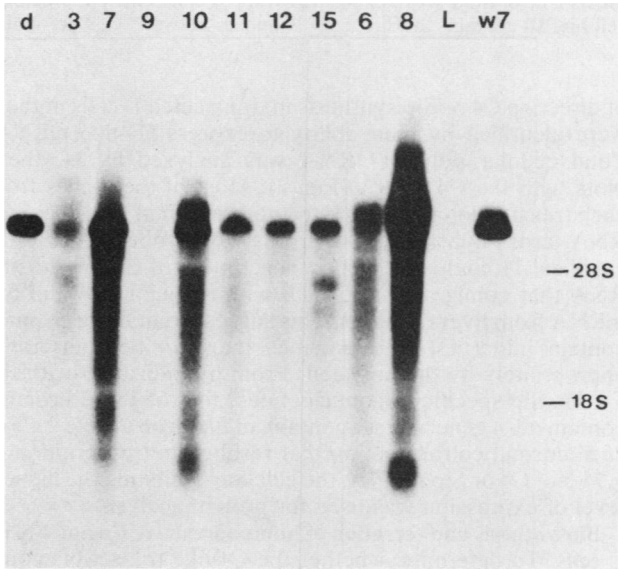

FIG. 3. Expression of C4 and Slp mRNA in L-cell cotransformants. Total cellular poly(A)$^+$ RNA was isolated from Ltk^+ cell lines, as described in Materials and Methods. This RNA was run on Northern blots as described in the text and hybridized to nick-translated C4 cDNA. The lanes are numbered in accord with the transfected cell line number (see Table 1). The first and last lanes contain 0.5 μg of female liver poly(A)$^+$ RNA from B10.D2 (lane d) or B10.W7R (lane w7) mice, respectively. The other lanes, derived from L-cell RNAs, all contain 5 μg of poly(A)$^+$ RNA, except lanes 12, 6, and 8, which each contain 1 μg of poly(A)$^+$ RNA. The L lane contains 10 μg of poly(A)$^+$ RNA from Ltk cells. Autoradiography was for 16 h.

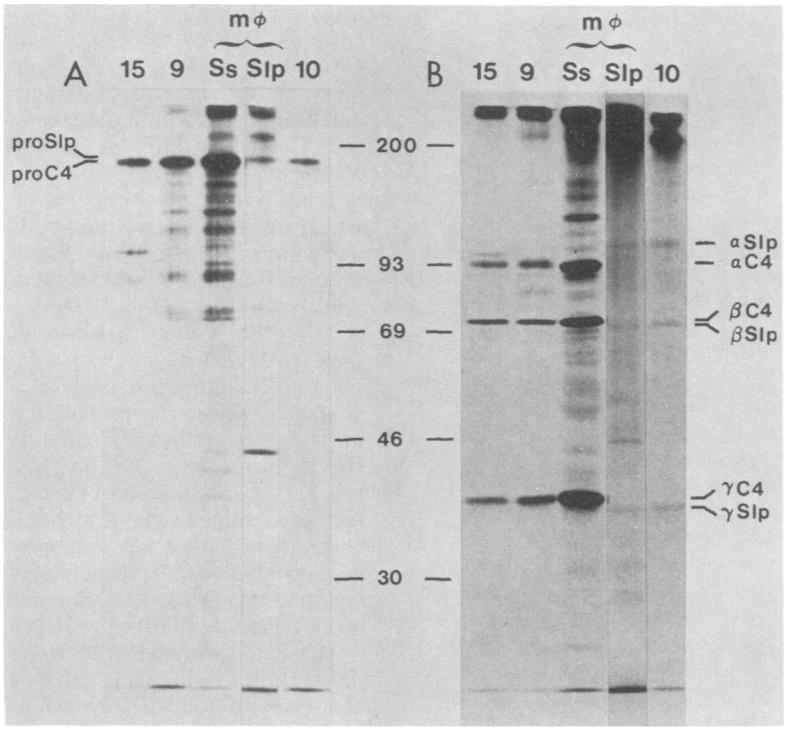

FIG. 4. Biosynthesis and secretion of [^{35}S]methionine-labeled C4 and Slp. Cells were cultured and labeled and media and cell lysate samples were immunoprecipitated as described in Materials and Methods. For this gel, all samples were incubated with rabbit anti-Ss serum, except the macrophage (mφ) Slp sample which was incubated with mouse anti-Slp serum. (A) Immunoprecipitates of cell lysates; (B) protein immunoprecipitated from extracellular media. All samples were electrophoresed on the same 10% sodium dodecyl sulfate-polyacrylamide gel, but panel A is from a 1-day exposure and panel B is from a 1-week exposure. The macrophage lane of panel B required a 6-week exposure, possibly owing to the thermolability of processed Slp and the weaker affinity of the anti-Slp antibody. The Slp-specific bands can be seen in the Ss lane of the 1-week exposure owing to more efficient precipitation with the less-specific antiserum. Cell lines 15 and 9 contain C4 genes; cell line 10 contains an Slp gene. Numbers between gels are molecular weight ($\times 10^3$) markers.

of directing C4 or Slp synthesis in transfected L cells initially were identified by their ability to express C4-like mRNA. Total cellular poly(A)$^+$ RNA was analyzed by Northern blots with the C4 cDNA (Fig. 3). Most of the clones from each transfection that contained donor cosmid DNA showed RNA cross-hybridizing with the C4 probe. Of the 15 cosmids, 11 could direct the transcription of C4-hybridizing RNA that comigrated at 5.7 kb with authentic C4 and Slp mRNA from liver (Table 1). This suggests that these cosmids contain intact C4 or Slp genes that can be transcribed appropriately by the host cell. From hybridization with the C4- or Slp-specific oligonucleotides, five of these cosmids contain a C4 gene and six contain an Slp gene (Table 1). For each cosmid cotransfection that resulted in transcription of 5.7 kb of C4 or Slp mRNA, the cell line exhibiting the highest level of expression was used for protein analysis.

Biosynthesis and secretion of immunoreactive C4 and Slp by L cells. To determine whether the C4-like transcripts could be translated into pro-C4 and pro-Slp molecules capable of being processed and secreted by L cells, biosynthetically radiolabeled cellular and secreted proteins were characterized. Cell lysates and extracellular media were reacted with rabbit anti-Ss sera, which recognizes both C4 and Slp, or mouse anti-Slp sera that is specific for Slp. Cells containing a 5.7-kb C4 or Slp mRNA also produced immunoprecipitable pro-proteins of approximately 200,000 molecular weight, as in peritoneal macrophages (Fig. 4A), whereas L cells themselves did not (data not shown). Pro-Slp was detectably larger than pro-C4.

Transfected L cells are capable of processing the precursors and secreting them into the media where they are found correctly cleaved into α, β, and γ chains of sizes similar to those found in macrophages (22). Cell lines 15 and 9 both contained C4 genes and secreted proteins with chain sizes of 95,000, 74,000, and 34,000 molecular weight. Processed Slp is readily distinguished from C4 owing to significant size differences in the peptide chains. The α chain of Slp is 105,000 molecular weight, which is larger than the C4 α chain. The Slp β and γ chains are somewhat smaller than their C4 counterparts, having molecular weights of 72,000 and 32,000, respectively. Because the Slp molecule is thermolabile, its accumulation in medium of L cells is noticeably less than for C4. To further verify the identity and authenticity of these proteins, we performed a radioimmunoassay (RIA) for Slp (7) on media from nine cotransfected L-cell lines, each containing a different cosmid DNA, as well as on Ltk$^-$ control cells (Table 1). Two different monoclonal antibodies directed at separate epitopes on the Slp molecule both detected significant Slp levels in the media of four cell lines containing Slp^{w7} genes, but not in media from Ltk$^-$ cells or any of the cell lines with foreign C4 genes. Cell line 3 is a low expressor of Slp, as seen by its mRNA level (Fig. 3) and a faint pro-Slp band on protein gels (data not shown). Therefore, the RIA activities of cell lines 3 and 11 (C4 expressing) are considered not significantly above background in this assay.

Distinct Slp^{w7} genes are capable of expressing polypeptides. Although multiple C4/Slp genes exist in the B10.W7R

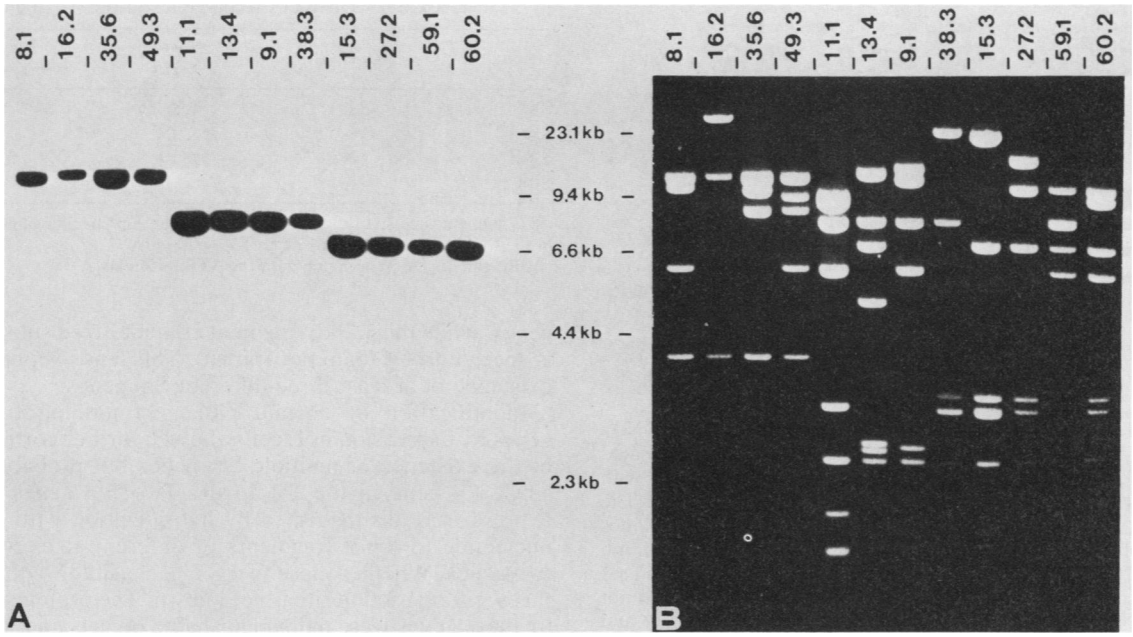

FIG. 5. Hybridization of *Sma*I-digested cosmid clone DNAs. A 300-ng sample of B10.W7R cosmid clone DNAs, indicated above each lane, was digested with *Sma*I, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose. (A) Hybridization of the filter to both the C4- and Slp-specific oligonucleotides, from the COOH terminus of the C4a peptide, which were ^{32}P end labeled to 2×10^8 cpm/μg. Hybridization of identical filters with either the C4 or the Slp probe shows the C4 probe hybridizing only to the 11-kb band in the first four lanes and the Slp probe hybridizing only to the 8- and 6.5-kb bands of the eight right lanes. Thus, the first four cosmids on the left contain C4 genes; the other eight cosmids shown contain Slp genes. (B) Photograph of the gel stained with ethidium bromide before transfer. Numbers between gels show molecular weight markers.

mouse, protein heterogeneity has not been noted. Additional C4 or Slp genes could be substantially similar with respect to their protein product or could represent pseudogenes or only partially duplicated genes. Of particular interest is the possibility of similar genes that express but are differentially regulated. To assess the number and expression of different C4 and Slp genes in the *w7 S* locus, we examined structural gene differences of the cosmid clones in conjunction with the transfection data. Since most of the restriction site differences between the cosmid clones relate to the extent of their inserts, we more rapidly scanned for heterogeneity by probing Southern blots of cosmid DNAs.

Initial characterization of multiple Slp genes derives from restriction site differences and hybridization studies with the C4- and Slp-specific oligonucleotide probes. Figure 5 shows a *Sma*I digestion difference among the Slp clones. Of the eight Slp cosmid clones transfected into L cells, four showed hybridization with a 6.5-kb *Sma*I fragment and four hybridized with an 8-kb *Sma*I fragment (Fig. 5A). All four of the cosmid clones that contained the 6.5-kb *Sma*I fragment were capable of expressing Slp after transfection into L cells (Table 1). Of those clones containing the 8-kb *Sma*I fragment, two expressed Slp and two did not show a 5.7-kb mRNA. These eight cosmids are independent isolates, as seen by their unique *Sma*I digestion patterns on the gel before transfer (Fig. 5B). The difference in the size of the hybridizing fragment in the two groups of clones is not due to differential extents of recombinant insert, because cosmid vector does not hybridize to this band (data not shown). The cosmids that express C4 after transfection into L cells, and one of the nonexpressing C4 cosmids, had an 11-kb *Sma*I fragment that hybridized to the C4 oligonucleotide (Fig. 5A). The remaining nonexpressing C4 cosmid showed no hybridization with this probe and was presumably missing much of the 5′ portion of the gene (data not shown). Thus, the *Sma*I restriction pattern can distinguish two different and expressible cloned Slp genes, but fails to detect a heterogeneity within C4 genes. The heterogeneity of *Sma*I sites within Slp^{w7} genes cannot be shown directly in the genome because these sites are methylated in vivo.

A further heterogeneity within Slp^{w7} genes was seen after digestion with the enzyme *Hin*dIII and can be substantiated by comparison with a genomic digest of DNA from the congenic B10.W7R strain. *Hin*dIII digests of cosmid DNAs from five C4-containing cosmid clones (Fig. 6, on the left of lane w7) were probed with a 3.6-kb C4 cDNA. All the C4 clones had a 15-kb *Hin*dIII fragment as seen in the *w7* genomic digest (lane 6). All the C4-expressing clones also had the 4.8-kb *Hin*dIII fragment, whereas cosmid 8.1 did not and thus may not express owing to the absence of requisite 3′ information. Of the eight Slp clones (on the right of lane w7), only cosmids 11.1 and 13.4 did not express in L cells. Five of the cosmids that did express showed a 19-kb *Hin*dIII fragment that also hybridized intensely in the genomic digest. At least two different Slp genes contain this fragment in the genome, since cosmid 38.3 had the 8-kb *Sma*I fragment, whereas the other four were characterized by the 6.5-kb *Sma*I fragment (Fig. 5). Three additional *Hin*dIII fragments that occurred in the genomic digest (of 23, 11, and 6.6 kb) were found in cosmid 11.1, which does not express in L cells. Whether cosmids 13.4 and 9.1 represent another Slp gene(s) or appear different owing to the limits of the recombinant insert has not yet been determined. A summary of the distinct S^{w7} genes is presented in Table 2. This analysis reveals two distinguishable Slp genes that are capable of expression in L cells and a third Slp gene that may lack

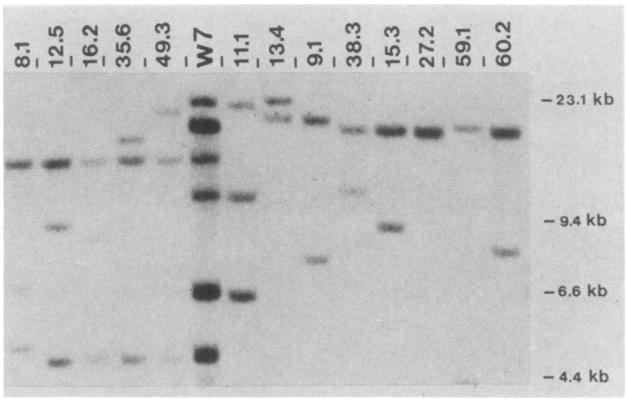

FIG. 6. Hybridization of *Hin*dIII-digested cosmid clone DNAs and B10.W7R liver DNA. Each cosmid DNA (25 pg) (indicated above each lane) and salmon DNA (15 μg) were digested with *Hin*dIII, as well as 15 μg of B10.W7R liver DNA (lane w7). The cosmid DNAs are arranged in the same order as in Fig. 5 (C4 clones to the left of the w7 lane, Slp clones to the right), with the addition of cosmid 12.5 which expresses C4 after transfection. C4 cosmid 8.1 does not express in L cells; Slp cosmids 11.1 and 13.4 also do not express. DNAs were electrophoresed on a 0.5% agarose gel and transferred to nitrocellulose, and the filter was hybridized to a 3.6-kb C4 cDNA extending from the 3′ terminus of the mRNA. Numbers on the right show molecular weight.

requisite regulatory information in cosmid 11.1 or may represent an Slp pseudogene in the S^{w7} locus.

DISCUSSION

The variety of regulatory alleles for C4 and Slp that exist in congenic mouse strains make the *S* locus an attractive system for studies of gene expression and evolution in addition to complement structure and function. Previous studies (4, 5) have described *S*-region genes from an *H-2*^{d} mouse (C4^h, Slp^a) and have shown that this C4 allele leads to production of hemolytically active C4 after transfection into mouse L-cell fibroblasts. In this report, we present evidence that the *S* locus of the B10.W7R mouse (C4^h, Slp^{w7}; constitutive rather than sex-limited expression of Slp) contains multiple C4/Slp genes and that both proteins can be expressed and processed after gene transfer of isolated cosmid clones. Introduction of these genes into heterologous cells may be the most straightforward means to determine exactly how many different and (potentially) expressible C4 and Slp genes are present in this mouse strain, since the large size and extensive homology of these genes would otherwise require inordinate molecular characterization. Further, transfection into hormonally responsive host cells may determine if multiple Slp genes are under similar or different regulation.

That the S^{w7} locus contains multiple C4/Slp genes is indicated by additional and more intensely hybridizing fragments relative to other haplotypes on genomic Southern blots (Fig. 1). Quantitative blot hybridizations of Levi-Strauss et al. (16) show single C4 and Slp genes in most mouse strains, except those with testosterone-independent expression of Slp in which multiple C4/Slp genes are found. In support of this notion, at least four different S^{w7} genes were indicated by restriction site polymorphisms in isolated cosmid clones. Identification of the cosmid inserts as C4 or Slp by expression in L cells or by hybridization to the C4- or Slp-specific oligomers revealed that the 0.9-kb *Sma*I fragment found in the three cosmids of Fig. 2 is present in Slp genes, while the 3.7-kb fragment characterized subsequently is specific to C4 (data not shown). This lends support to the existence of at least three different Slp genes.

TABLE 2. Distinct C4/Slp genes in the S^{w7} locus

Gene	*Sma*I[a] (kb)	*Hin*dIII[b] (kb)	Representative cosmid clone
C4^{w7}	11	4.8, 15	35.6
SlpA^{w7}	6.5	19	27.2
SlpB^{w7}	8	19	38.3
SlpC^{w7}	8	23, 11, 6.6	11.1

[a] Fragment size hybridizing with the C4- or Slp-specific oligonucleotide probe to the COOH terminus of the C4a peptide.
[b] Fragment size hybridizing with the 3.6-kb C4 cDNA.

Identification of cosmid clones containing full-length genes by expression in L cells allowed further corroboration of the existence of multiple Slp genes but probably only a single C4 gene in the S^{w7} locus. Two Slp genes that can express were distinguished by hybridization with an oligonucleotide to *Sma*I fragments of different sizes within the two genes. Whether these two Slp genes differ by more than this *Sma*I restriction site is not known. The proteins encoded by these genes were indistinguishable on gels and were both recognizable by using three different Slp-specific antibodies. Thus, the genes are unlikely to differ significantly at least in regions encoding Slp-specific epitopes and cleavage sites.

The two Slp genes that can express in L cells share in common a 19-kb *Hin*dIII fragment. A third Slp gene shows a different *Hin*dIII restriction pattern that correlates with additional bands in the *w7* genome. The cosmid containing this gene does not express in L cells and so may be lacking necessary transcription signals. Alternatively, it may represent a pseudogene in the B10.W7R mouse. While the 23-kb *Hin*dIII fragment of cosmid 11.1 is similar in size to the Slp-specific fragment in the *H-2*^{d} mouse (Fig. 1), the other fragments of these two Slp genes do not coincide.

The hybridization pattern for the *Hin*dIII digest of *H-2*^{w7} DNA can be accounted for by a minimum of four different C4/Slp genes, each specific to the *w7* haplotype (Table 2). We have no evidence as yet for more than one C4 gene in the *w7* genome. Both *H-2*^{d} and *H-2*^{w7} C4 genes are C4^h alleles, yet differ in their *Hin*dIII restriction sites. This may relate to structural differences in the two C4 proteins resulting in the lower hemolytic efficiency of C4 from *H-2*^{w7} mice (2). The distinct Slp^{w7} genes may now be distinguished as in Table 2 as SlpA, SlpB, and SlpC. Further molecular mapping and overlapping of these cosmid clones should elucidate the physical arrangement of these genes and be informative for mechanisms of gene duplication. It is striking that constitutive expression of Slp correlates not just with multiple C4/Slp genes (16) but, at least for the B10.W7R strain, with apparent amplification of just the Slp gene. It will be interesting to determine whether the products of multiple and recent (16) gene duplications still all reside within the *S* locus, and whether any partial gene copies or hybrid C4/Slp genes are present.

That both C4 and Slp can be expressed, processed, and secreted by mouse L-cell fibroblasts indicates that the enzymatic machinery necessary for cleavage and modification of these proteins may be constitutively expressed in a variety of cells. Thus, expression of individual genes by transfection may allow protein differences to be detected and ascribed to different S^{w7} genes. Transfections into hormonally responsive cell lines should also be useful in localizing DNA sequences underlying the differential regulation of Slp^a and

Slp^{w7} alleles. Furthermore, it is conceivable that distinct Slp^{w7} genes are themselves regulated differently. In vivo, an Slp^{w7} gene that was in fact regulated by testosterone might not be detected above high constitutive expression of Slp in both sexes. For genes as large and homologous as C4 and Slp, transfection studies provide a functional assay for localizing DNA sequence differences of structural or regulatory significance or both more rapidly and definitively than structural gene comparisons alone.

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