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Genetics

Multiple duplications of complement *C4* gene correlate with *H-2*-controlled testosterone-independent expression of its sex-limited isoform, C4-Slp

(mouse cDNA clones/gene expression/DNA sequence analyses/gene duplications)

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ABSTRACT **Mouse liver cDNA clones related to the C4 and C4-Slp isoforms of the fourth component of complement differ by few nucleotide changes within a region of substantial divergence from human C4. It is suggested that the mouse *C4* gene duplication is an evolutionarily recent event with respect to the time of mammalian radiation. This conclusion is reinforced by the presence of a single *C4* gene in the Syrian hamster. Most *H-2* haplotypes, including those characterized by an undetectable C4-Slp protein, possess two *C4* gene copies which, in contrast to the neighboring factor B, show a marked restriction site polymorphism. The genetic variation of this region is emphasized by the presence in the mouse of a rare "polymorphism" for *C4* gene number. Multiple *C4*-related gene copies characterize those exceptional wild-derived *H-2* haplotypes, $H\text{-}2^{w7}$, $H\text{-}2^{w16}$, and $H\text{-}2^{w19}$, that determine the expression of the C4-Slp protein in female animals.**

The *S* region of the mouse *H-2* gene complex was defined by two nonallelic genes encoding the isotypic forms C4 and C4-Slp (sex-limited protein)§ of the fourth component of the complement system (1–3).

Prior to the discovery of their biological function and the recognition that discrete genes encode the C4 and C4-Slp forms, the two proteins were collectively referred to as the mouse Ss (serum substance) protein, detected by means of xenoantisera (see ref. 4 for a recent review). C4-Slp, the form that is identified by an allotypic antiserum, does not possess all the functional properties of C4 and in fact is hemolytically inactive. The two proteins display considerable structural and quantitative genetic variation (5) and are also distinguished by the regulation and site of expression of their genes. C4-Slp is normally produced only in hepatocytes and its expression is controlled by at least two genetically dissectable mechanisms. By definition, this protein displays sexual dimorphism in that it is androgen controlled and normally undetectable (although testosterone inducible) in females of the standard C4-Slp-positive strains. However, some exceptional wild-derived *H-2* haplotypes—namely, $H\text{-}2^{w7}$, $H\text{-}2^{w16}$, and $H\text{-}2^{w19}$—determine the expression of C4-Slp in both male and female mice (6, 7). In these mice, the protein is produced not only by the liver but also by resident peritoneal macrophages, a cell type that in all strains is the second normal site of C4 expression. This allelism for an androgen-controlled or androgen-independent C4-Slp expression was shown by segregation analysis to involve *cis*-acting regulatory elements genetically inseparable from the structural gene (8). Alternatively, C4-Slp can be expressed in female mice of certain strains by a second type of androgen- and androgen receptor-independent mechanism, which, however, is not linked to the *H-2* complex. This latter phenotype appears to be controlled by *trans*-acting and as yet unmapped regulator genes (*rsl*) able to mimic testosterone action (9). On the other hand, the genetic bases of the lack of C4-Slp even in the males of strains carrying certain *H-2S* regions (e.g., $H\text{-}2S^k$, $H\text{-}2S^f$, $H\text{-}2S^b$, and $H\text{-}2S^q$), remain unresolved. The failure to detect the C4-Slp protein in these strains could be due to an untranscribed allele, an antigenically undetectable product, or the absence of the structural gene.

To define the mechanisms responsible for the diverse modes of expression of the C4 isotypes, we undertook the isolation of mouse C4 cDNA clones and in an initial analysis probed the molecular organization of the *S* regions of some of the appropriate *H-2* haplotypes.

MATERIALS AND METHODS

Extraction of Nucleic Acids. DNA was isolated (ref. 10, pp. 280–281) from liver nuclei prepared by the citric acid method. RNA was isolated essentially as described by Chirgwin *et al.* (11) and precipitated in 2.7 M lithium chloride.

Molecular Cloning and Nucleotide Sequence Analysis. Sucrose gradient fractions in the 28S region were assayed for the presence of C4 mRNA by *in vitro* translation. C4-enriched fractions were pooled and double-stranded cDNA synthesis was performed according to published procedures (ref. 10, pp. 212–246). Electrophoretically fractionated cDNA larger than 1.5 kilobases (kb) was annealed with the *Pst* I-cleaved pBR322 plasmid, using the dC·dG homopolymer method. Upon transformation of the *Escherichia coli* strain 5K, 5×10^3 to 10^4 bacteria were plated on Pall-Biodyne filters supported by tetracycline-containing agar. Segments of cDNA insertions were subcloned in the M13 phages mp8 or mp9 and sequenced (12) by using the kit provided by Amersham.

DNA and RNA Blots and Hybridizations. Agarose gel electrophoreses, blotting of DNA or RNA, and hybridizations with cDNA probes were performed according to standard methods (ref. 10, pp. 150–163, 382–389, and 202–203).

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Abbreviations: C4, fourth component of complement; Slp, sex-limited protein; bp, base pair(s); kb, kilobase(s) or kilobase pair(s).
§We feel that the commonly utilized nomenclature of C4 and Slp emphasizes the physiological divergences of the two genes and it obscures their isotypic relationship. Here, following a convention in use for other isotypes, we adopt a common prefix in the designation of the two genes, to render immediately obvious their relatedness as products of a recent gene duplication.

RESULTS

C4 cDNA and Genomic Clones. A cDNA library was prepared with C4-enriched fractions of liver RNA from the mouse strain B10.W7R, which produces relatively high levels of C4 and expresses C4-Slp in a testosterone-independent fashion. Approximately 5000 clones were screened by using the insertion of the human C4 cDNA clone pAlu/7 (13). We have determined the nucleotide sequence of a portion of plasmid pMC4/7 encoding the α-chain carboxylterminal half (Fig. 1*B*). The sequence specifying an arginine tetrapeptide (nucleotides 1018–1029) makes it possible to align precisely the pMC4/7 insertion with respect to the C4 precursor protein, since this tetrapeptide identifies the α–γ junction (14, 16).

The insertion of our clone pMC4/7 includes the 458-bp cDNA sequence of the previously described murine C4 clone pMLC4/W7-2 (14), from which it differs only by one nucleotide residue of the 270 that have been compared. The portion of the murine C4 α-chain shown in Fig. 1 is 79% homologous to the corresponding nucleotide sequence of human C4A (15). A striking difference between the murine and the human sequence in the C4-α_4 region is the presence in the former of three additional nucleotides (residues 931–933 in Fig. 1). Interestingly, in the same region of C4-α_4 we have also found an exceptional cluster of nucleotide substitutions (unpublished data) between the sequence of pMC4/7 and that of pMC4/201, a different murine C4 cDNA clone previously related to the C4-Slp isotype (17). Noticeably, the amino acid sequences deduced from the murine cDNA clones fail to reveal any correspondence between the human and murine isotypes. In particular, within the C4d region, which contains a cluster of four sites of isotypic variation in man (15), two murine amino acid residues, encoded by clone pMC4/7, are related to the human C4A sequence, whereas the other two are related to the C4B sequence (Fig. 1).

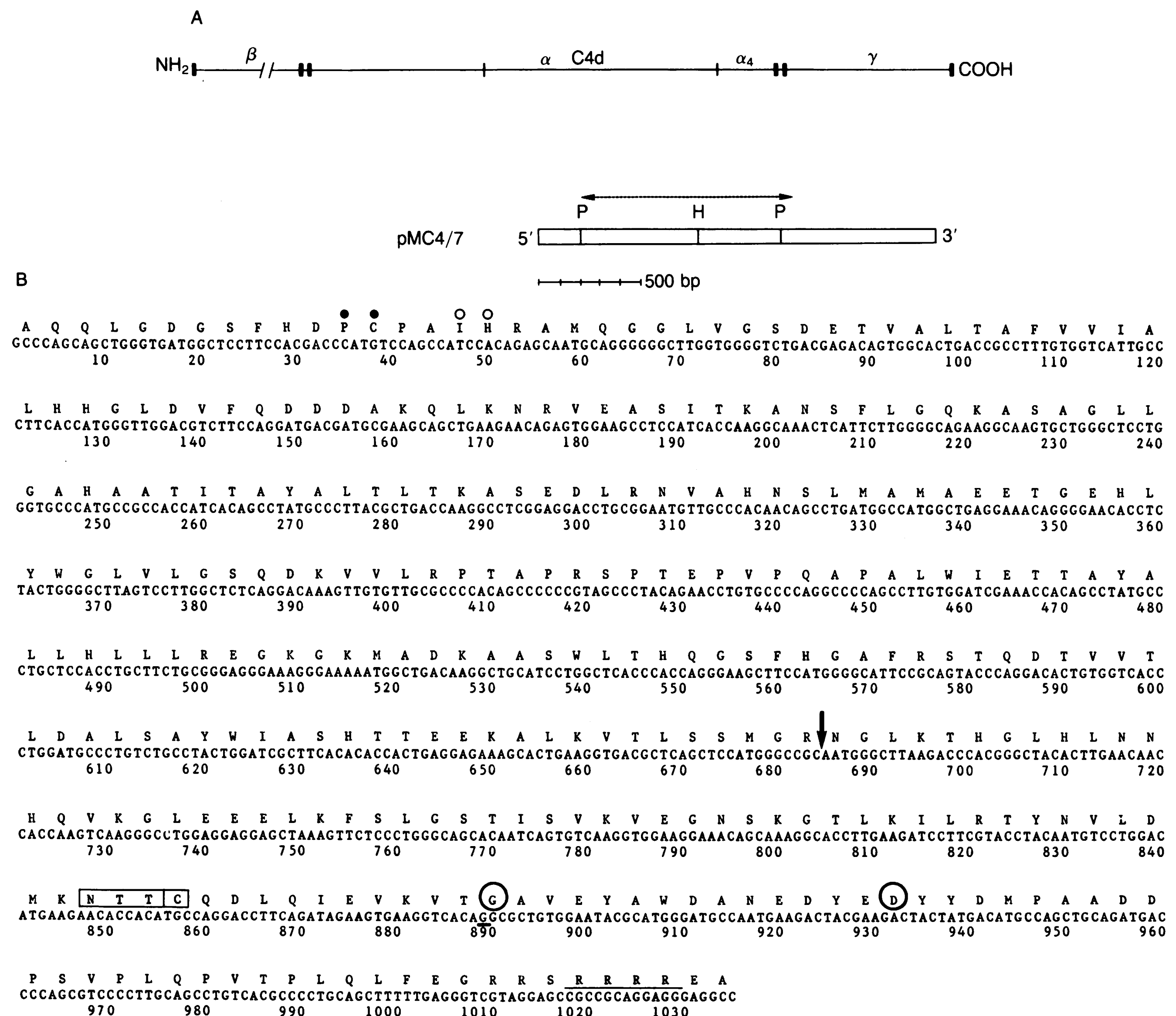

FIG. 1. (*A*) The *Pst* I insertion of cDNA clone pMC4/7 is aligned with the structure of the pro-C4 protein (top line). Restriction sites are *Pst* I (P) and *Hin*dIII (H). bp, Base pairs. (*B*) The nucleotide sequence is derived from the region of pMC4/7 between the arrowheads in *A*. The four arginines underscored at the 3′ end of this sequence correspond to the C4 α–γ junction. An arrow indicates the junction of peptides C4d and C4-α_4. The potential site of N-linked glycosylation and the adjacent cysteine residue of the α_4 fragment (14) are boxed. A glycine that replaces a serine of the previously described murine sequence (14) is encircled and the substituted nucleotide is underlined. Also encircled is an aspartic acid that apparently represents an insertion with respect to the human C4A sequence. Four sites where amino acid replacements distinguish the human C4A and C4B protein (15) are marked: ● and ○ indicate identity of the murine residues with C4A and C4B, respectively.

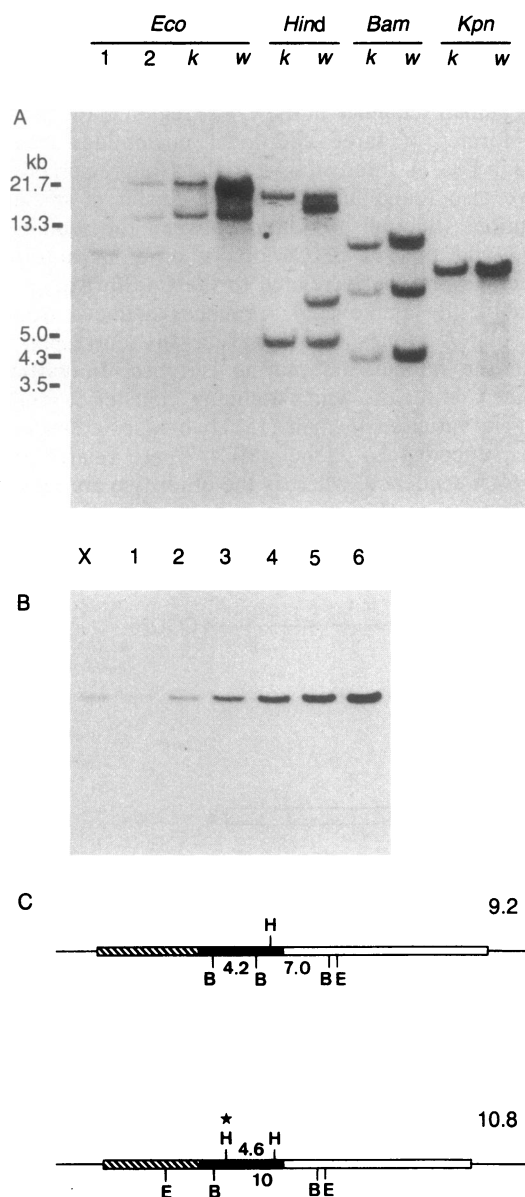

FIG. 2. *(A)* Hybridization of the 3′-most *Pst* I fragment of the pMC4/7 insertion to DNA of mouse-hamster hybrid cell lines (lanes 1 and 2) or to DNA of mouse strains B10.HTT (lanes *k*) and B10.W7R (lanes *w*). Thirty micrograms of hybrid cell line DNA or 15 μg of mouse liver DNA was digested with the indicated enzymes and electrophoresed on a 0.8% agarose gel. Hybrid cell lines of lanes 1 and 2 differ by absence or presence of the mouse chromosome 17, respectively. *(B)* Quantitative Southern blot hybridization. Ten micrograms of authentic B10.HTT DNA (lane X) or appropriate amounts of 10.8 cosmid clone DNA supplemented with 10 μg of *E. coli* carrier DNA (lanes 1–6) were digested with *Kpn* I and hybridized with the same probe used in *A*. Lanes 1–6 contain the amounts of cloned DNA corresponding to 1–6 gene equivalents, respectively. *(C)* Scheme of the two C4 genes cloned from the AKR strain ($H\text{-}2^k$). The arms of the cosmid vector pTL5 are represented with thin lines. Filled bars, 8.4-kb *Kpn* I fragments containing exons homologous to the insertion of pMC4/7. Hatched bars, 5′ portions of the genes; the precise 5′ boundaries have not yet been determined. Only the restriction sites relevant for the interpretation of the DNA blots are indicated: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. The star indicates the distinctive site in 10.8. Restriction fragment length is indicated in kb.

Both the insertion of the human C4 clone pAlu/7 and fragments derived from the murine clone pMC4/7 were used to screen a cosmid library of mouse AKR liver DNA ($H\text{-}2^k$) (18). Two distinct C4 genomic clones, 9.2 and 10.8, are depicted in Fig. 2*C*; their orientation with respect to the C4

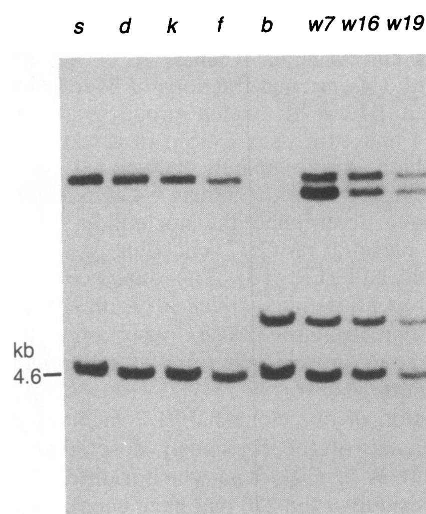

FIG. 3. Hybridization of *Hin*dIII-digested mouse liver DNA with the C4 probe described in Fig. 2. The *S* region alleles indicated above each lane are those of the following strains: B10.S(7R) (*s*); B10.S(9R) (*d*); B10.HTT (*k*); B10.M (*f*); C57BL/10 (*b*); B10.W7R (*w7*); B10.BUA1 (*w16*); B10.KPB128 (*w19*).

mRNA was established by hybridization with 5′ and 3′ probes derived from pMC4/7. Note that the presence of a *Hin*dIII site marked by a star characterizes clone 10.8 with respect to clone 9.2. The cDNA clones pMC4/7 and pMC4/201, related to C4 and C4-Slp respectively, also differ by a *Hin*dIII site (17). We have determined the nucleotide sequences of cosmid 10.8 around this *Hin*dIII site, and we found exonic sequences corresponding to those of cDNA clone pMC4/7 (data not shown). Thus, a *Hin*dIII site appears to mark an isotypic variation in the coding sequences of the *C4* and *C4-Slp* genes, at least in $H\text{-}2^k$ and $H\text{-}2^{w7}$ strains. However, the finding of a 4.6-kb *Hin*dIII fragment in the C4 DNA blots of these strains (Fig. 2*A* and ref. 19), as well as in those of all other strains so far studied (Fig. 3 and unpublished observations), suggests that the presence of this *Hin*dIII site is a general marker of the murine C4 isotype.

Two Genes Characterize the *C4* Region of the Common *H-2* Haplotypes. We initially used a number of restriction endonucleases in an effort to detect nucleotide sequence variation in the *H-2S* region of the congenic strains, B10.HTT and B10.W7R, since they differ considerably in their C4 phenotypes. The former is C4 "low" and C4-Slp negative, while the latter is C4 "high" and expresses C4-Slp in large amounts in a testosterone-independent fashion. Surprisingly, the *C4* genes appeared to be amplified in the wild-derived haplotype of B10.W7R mice (Fig. 2*A*). The specificity of hybridization of the C4 cDNA probe was monitored in lanes 1 and 2, which contain *Eco*RI-digested DNA of mouse (BALB/c)–hamster hybrid cell lines, one of which (lane 1) has retained at least one copy of all mouse chromosomes except chromosome 17, which is absent. Both cell hybrids contain about twice as much nuclear DNA as a normal mammalian cell (20). The intensity of hybridization of the mouse DNA fragments in the second lane is consistent with the presence, proven cytogenetically, of only one copy of mouse chromosome 17 in the second cell line. The single *Eco*RI fragment common to both lanes indicates the presence of only one *C4* gene in the haploid genome of the hamster. This conclusion is corroborated by the finding that hamster DNA digested with a number of restriction enzymes exhibits a single fragment hybridizing with short C4 probes (data not shown).

A quantitative blot hybridization (Fig. 2*B*) was performed with *Kpn* I-digested B10.HTT DNA, since this enzyme yields from each C4 genomic clone (Fig. 2*C*) an 8.4-kb

fragment that contains all the sequences hybridizing with the C4 cDNA probe. From the intensity of hybridization in lane X and the isolation of two distinct genomic clones, we conclude that two genes characterize the *C4* region of $H\text{-}2^k$ mice. The Southern hybridization in Fig. 2*A* reveals a 4.6-kb *Hin*dIII fragment diagnostic of the *C4* gene and a very large fragment representing the *C4-Slp* copy of the $H\text{-}2^k$ genome. Similar *Hin*dIII fragments indicating the presence of two genes are found in three other common haplotypes, bearing the *S* region alleles S^s, S^d, and S^f (Fig. 3). Note that these strains differ in their C4 phenotypes. In particular, the S^f allele, like the S^k allele, fails to express C4-Slp, while the S^s and S^d alleles are prototypes for normal expression of both C4 isoforms. A fifth common haplotype, which bears the S^b allele (Fig. 3, lane b), also appears to carry two *C4*-related genes, one of which, presumably *C4-Slp*, displays a *Hin*dIII site polymorphism (see also ref. 19). This *Hin*dIII pattern is also obtained with mice of the $H\text{-}2^q$ haplotype (DBA/1 strain, not shown).

That the *S* region of most common haplotypes can be characteristically distinguished by restriction endonuclease site polymorphism is shown in Fig. 4. While polymorphism of *Bam*HI sites can be exploited to distinguish *f* from *k* or from *d S* region alleles (Fig. 4*A*), even the latter can be discriminated (Fig. 4*B*) by using a *Hin*dIII site polymorphism revealed by a cDNA probe 5′ to the *Hin*dIII site of pMC4/7 (see Fig. 1).

The C4 mRNA levels of strains and sexes differing in C4 or C4-Slp expression were compared by blot analysis (Fig. 5). Hybridization to the 28S ribosomal RNA can be used as an internal quantitative control. This hybridization is clearly due to an interaction between the dG·dC tails of the probe and the 28S ribosomal RNA, since it was not observed either by using the corresponding poly(A)$^+$ RNAs or by hybridizing total RNA with an internal fragment of the same cDNA insertion (data not shown). Liver C4 mRNA concentrations (see also refs. 14 and 19) correlate with the C4 serum levels for the B10.HTT ($H\text{-}2S^k$) and the B10.S(9R) ($H\text{-}2S^d$) strains (lanes 1 and 2, respectively) since the former is characterized by low levels of serum C4. The C4 mRNA concentration in liver of male C3H.W7 mice is surprisingly high (lane 6), even taking into account the elevated C4 and C4-Slp serum levels in this strain. The different C4 levels in the female and male

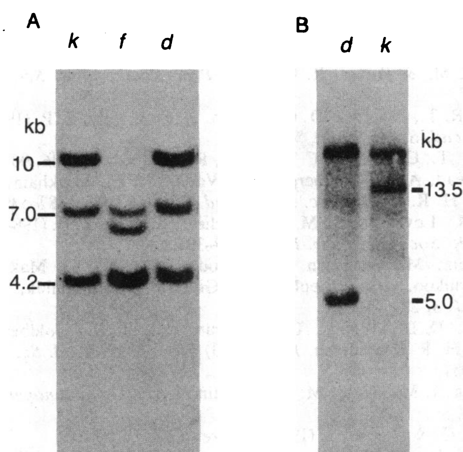

FIG. 4. Restriction site polymorphism of the murine *S* region. Letters above each lane indicate the *H-2S* allele. The corresponding strains are listed in Fig. 3. (*A*) DNA was digested with *Bam*HI and hybridized with the two large *Pst* I fragments of the pMC4/7 insertion. (*B*) DNA was digested with *Hin*dIII and hybridized with the larger (5′) *Pst* I/*Hin*dIII fragment. Note that the polymorphic *C4* gene fragments of 5.0 and 13.5 kb are not detected by the more 3′ probe used in Fig. 3.

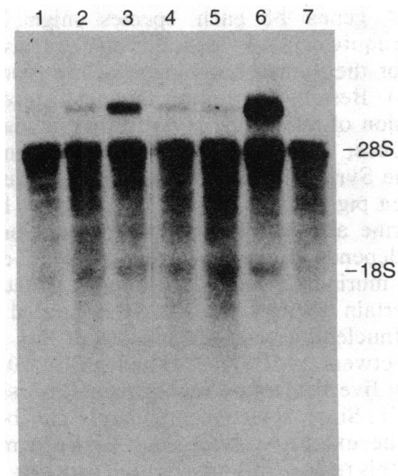

FIG. 5. Electrophoretic separation of total liver RNA (15 μg) on a 1.2% agarose/3% formaldehyde gel and hybridization to the 3′-most *Pst* I fragment of the pMC4/7 insertion. Lane 1, B10.HTT (male); lane 2, B10.S(9R) (female); lane 3, B10.S(9R) (male); lane 4, B10.G (female); lane 5, B10.G (male); lane 6, C3H.W7 (male); lane 7, C4-negative mouse hepatoma cell line (21). Total RNA was preferred because of the difficulty of quantitatively retaining C4 messengers in the poly(A)$^+$ fraction.

RNA of strain B10.S(9R) (lanes 2 and 3, respectively) must be accounted for, at least in part, by the male-restricted expression of the C4-Slp isotype in this strain. The higher steady-state levels of total male C4 mRNA may be taken as an indicator of C4-Slp gene transcription (19). The finding of similar mRNA levels in female (lane 4) and male (lane 5) B10.G ($H\text{-}2^q$) mice would then suggest that the *C4-Slp* gene of this strain is not transcribed.

The *H-2*-Controlled Testosterone-Independent Expression of C4-Slp Correlates with Multiple Gene Duplications. The nature of the *C4* gene duplication apparent in the lanes of Fig. 2 that contain B10.W7R DNA was examined in more detail. First, gene duplication(s) could have occurred during the transfer of the wild *w7* haplotype to the C57BL/10 background. Therefore, we examined the DNA of the C3H.W7 strain, which carries the same *S* region but on the C3H background genotype. The same hybridization pattern as B10.W7R was obtained (data not shown). This result indicates that the number of duplicated *C4* copies in B10.W7R mice is a property of the *S* region of this wild-derived *H-2* haplotype. To validate a possible correlation between the testosterone-independent mode of C4-Slp expression, a distinct feature of this haplotype, and the observed duplication of its *C4* genes, two additional *H-2*-congenic strains, B10.BUA1 and B10.KPB128, which are also characterized by the testosterone-independent production of C4-Slp, were examined. Their DNA reveals the characteristic four-banded *Hin*dIII pattern of B10.W7R (Fig. 3, lanes *w16* and *w19*). However, the intensity of hybridization of the second largest *Hin*dIII fragment suggests that the *S* region of B10.W7R mice contains at least one additional *C4*-related gene copy (lane *w7*) relative to the B10.BUA1 and B10.KPB128 mice. Thus, it appears that $H\text{-}2S^{w7}$ mice have undergone an additional round of gene duplication. Interestingly, the serum levels of C4-Slp in these mice are at least 10-fold higher than those in $H\text{-}2S^{w16}$ or $H\text{-}2S^{w19}$ (4).

DISCUSSION

The finding of two isologous forms of the C4 protein, both in mouse and man, and the recognition of a strong evolutionary stability in the genetic composition of the major histocompatibility complex have nourished the speculation that

the two *C4* genes of each species might be direct phylogenetic equivalents. Indeed, this concept has prompted the search for the human homologue of the mouse C4-Slp form (22, 23). Besides the notion that testosterone-dependent expression of a *C4* gene is not found in species other than the mouse, our observation that only one gene is present in the Syrian hamster, as probably is the case also for the guinea pig (16, 24), makes us favor the hypothesis that the murine and human *C4* gene duplications are the result of independent events. The degree of divergence between the murine *C4* and *C4-Slp* genes is astonishingly small for certain regions. In fact, the limited sequence comparison (nucleotide residues 460–816 of Fig. 1) that we have made between pMC4/7 (C4) and pMC4/201 (C4-Slp) revealed only five nucleotide replacements, of which three are silent (17). Since selective arguments can be rejected because of the extensive divergence between mouse and human C4 in this region, this observation supports the notion that the mouse C4 duplication is indeed a recent event. Such conservation is even more surprising in view of the questioned biological function of C4-Slp, and it should otherwise be explained by invoking some kind of sequence correction mechanism. Incidentally, the finding of two *C4* genes also in the rat (Lou strain; results not shown) does not permit us to date the mouse *C4* duplication with respect to the recent time of divergence of these two rodents.

The isolation of two types of mouse *C4* genes from a cosmid library of $H\text{-}2^k$ (18) and $H\text{-}2^d$ (19) strains is insufficient evidence to rule out a higher number of very similar gene copies. The results of our quantitative blot hybridization establish that the $H\text{-}2^k$ strain carries no more than two *C4*-related sequences per haploid genome. By analogy to $H\text{-}2^k$, most of the common *H-2* haplotypes appear also to carry two genes. This finding has important implications with regards to the genetic interpretation of at least three different expression variants of the mouse C4 isoforms. First, the low expression of C4 protein by the hepatocytes, but not the macrophages (25), of the $H\text{-}2^k$ mice, which fail to express C4-Slp, could be explained by invoking the differential activation of two distinct tissue-specific genes encoding the C4 isotype. However, the DNA blots shown in this paper and preliminary nucleotide data obtained from the AKR cosmid 9.2 clearly indicate that one of the two *C4*-related genes of $H\text{-}2^k$ has the sequence characteristics of a *C4-Slp* gene. Hence, the differential C4 levels in peritoneal macrophages versus hepatocytes of the $H\text{-}2^k$ strains must be due to the tissue-specific activity of regulatory sequences of one and the same *C4* gene. Similarly, the apparent dual mode of induction of *C4-Slp*, either by testosterone or by the uncharacterized autosomal mediators identified by the *rsl* mutation (9), cannot be due to the existence of two nonallelic *C4-Slp* structural genes. Finally, the failure of $H\text{-}2^f$, $H\text{-}2^b$, $H\text{-}2^k$, and $H\text{-}2^q$ strains to express C4-Slp must be explained not by the lack of the corresponding gene but by the existence of untranslated pseudogenes, or else they have so far escaped serological detection.

The C4 segment of the *H-2S* region is more polymorphic than its neighboring factor B locus (19, 26). Moreover, the genetic variability of the *C4* region is emphasized by the "polymorphism" in *C4* gene number that characterizes $H\text{-}2^{w7}$, $H\text{-}2^{w16}$, and $H\text{-}2^{w19}$, the only haplotypes known to determine a constitutive expression of the C4-Slp protein (6, 7). Although we do not know how many of the redundant genes are expressed, it is unquestionable that the change in topology of at least one *C4-Slp* gene was accompanied by a loss of its dependence on testosterone induction. As possible structural determinants of this change in the expression of a hormonally regulated gene, one can postulate either the direct effect of altered sequences neighboring the promoter region or the long-range influence of the DNA duplication on the packing of chromatin domains.

With three mutational events, the history of the mouse *C4* gene repeatedly underscores the significance of gene duplication and relocation in the evolution of gene activity. The DNA sequences that after the first gene duplication imposed testosterone dependence on a copy of the original *C4* gene appear to have been lost, perturbed, or superseded in coincidence with a second gene duplication event. Finally, the third round of gene duplication, through which the $H\text{-}2S^{w7}$ haplotype diverged from the $H\text{-}2S^{w16}/H\text{-}2S^{w19}$ common ancestor, again in all likelihood affected the transcriptional efficiency of at least one *C4-Slp* gene. In analogy to the disproportionate expression of a duplicated renin gene in the mouse (27), the higher level of the C4-Slp protein in B10.W7R mice (7) is not linearly correlated with the increase in gene copy number.

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