Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5061-5065, August 1983
Immunology

cDNA clone spanning the α–γ subunit junction in the precursor of the murine fourth complement component (C4)

(*H-2/S* region/differential hybridization/DNA sequence)

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Contributed by Donald C. Shreffler, May 10, 1983

ABSTRACT cDNA clones carrying parts of murine fourth complement component (C4, serum substance protein) mRNA sequences have been identified by differential hybridization to mRNA from a high C4-producing strain, B10.WR, and a congeneic low C4 strain, B10.BR, followed by hybrid-selected translation and DNA sequence analysis. One clone, pMLC4/w7-2, encodes an open amino acid reading frame that includes four tandem arginine residues immediately preceding a sequence 85% homologous with the NH_2-terminal sequence of the human C4 γ-chain. The amino acid composition of the predicted sequence upstream of the tandem arginines matches quite closely with the composition of a similar sized peptide at the COOH terminus of the human C4 α chain. The latter result raises questions regarding the nature and extent of plasma-mediated postsynthetic processing of the C4 α-chain COOH terminus. The results also demonstrate that strain differences in plasma C4 levels (low C4 vs. high C4) reflect differences in steady-state levels of liver C4 mRNA in these strains.

The S region of the murine major histocompatibility complex, *H-2*, was defined initially as a genetic locus controlling the quantity of the Ss (serum substance) protein in mouse serum (1). Ss has since been found to consist of two distinct proteins: C4, the murine fourth component of complement (2–4) and Slp (sex-limited protein), a protein that shares extensive structural homology with C4 but that has no complement activity and no known function (5, 6). Slp is distinctive in that, with some mouse strains, its expression depends directly on testosterone levels. Genetic analysis of polymorphic variants of C4 and Slp indicate that the S region harbors distinct structural genes for both proteins (7–12). As with the analogous region in the human *HLA* gene complex, the S region appears also to include the structural genes for complement components C2 and factor B (13, 14). These S region-encoded proteins constitute the class III products of the major histocompatibility complex.

As an initial step in examining the organization of the S region and the structure and expression of S region-encoded gene products, we have constructed cDNA clones from murine C4 mRNA. C4 is a glycoprotein composed of three disulfide-linked subunits, α, β, and γ, with M_rs of approximately 93,000, 78,000, and 33,000, respectively (see ref. 15 for a review). C4 biosynthesis takes place primarily in the liver; the protein is synthesized as a M_r 200,000 single-chain precursor, pro-C4, which is proteolytically processed to the heterotrimer prior to secretion (reviewed in ref. 16). The order of subunits in pro-C4 is β-α-γ (17–19). Here we describe a C4 cDNA clone that spans the α–γ junction in pro-C4. Carroll and Porter recently reported the isolation of a human C4 cDNA clone encoding a central portion of the human C4 α chain (20).

MATERIALS AND METHODS

Mice. Mice were bred and maintained in the Scripps animal facilities. Strain B10.WR came originally from the Washington University Department of Genetics mouse colony.

Cytoplasmic RNA. Fresh livers were homogenized on ice in 0.2 M NaCl/0.02 M $MgCl_2$/0.02 M Tris·HCl, pH 8.8 (10 ml per liver), with a motor-driven glass/Teflon homogenizer. After centrifugation at 600 × g for 5 min at 4°C, the supernatant was collected and added to an equal volume of freshly prepared proteinase K (Boehringer Mannheim) at 1 mg/ml in 2% $NaDodSO_4$/0.1 M NaCl/40 mM EDTA, pH 8. After incubation at room temperature for 20–30 min, the mixture was extracted twice with phenol/chloroform, 1:1 (vol/vol), extracted once with chloroform/isoamyl alcohol 24:1 (vol/vol), and precipitated with ethanol. This procedure yielded about 150–200 A_{260} units of nucleic acid per mouse liver.

Polyadenylylated RNA. Total cytoplasmic extract was fractionated by passage through poly(U)-Sephadex (Bethesda Research Laboratories) following the procedure recommended by the supplier. About 2% of the input eluted as poly(A)$^+$ RNA giving an overall yield of about 100 μg per liver.

Size-Selected RNA. Poly(A)$^+$ RNA was sized on linear 15–30% (wt/vol) sucrose gradients in 50 mM NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.2% $NaDodSO_4$. About 200 μg RNA in 0.2 ml of the same buffer without sucrose was heated at 65°C for 3 min, chilled on ice, applied to a 16-ml gradient, and centrifuged at 26,000 rpm in an SW 28.1 rotor (Beckman) for 15 hr at 20°C. RNA ≥28 S in size was collected and precipitated with ethanol; this fraction constituted 10–20% of the total poly(A)$^+$ RNA.

***In Vitro* Translation.** *In vitro* translations were performed with a commercially available ^{35}S reticulocyte lysate kit (New England Nuclear) by using the procedure described by the supplier with the following modifications: the RNA was denatured with 3 mM methylmercury hydroxide (MeHgOH) prior to translation (21) and the translation mix contained 1 unit of human placenta RNase inhibitor (22) (Biotec, Madison, WI) per ml. Ordinarily, 500 ng of RNA was translated in a 25-μl reaction. Translation products were immunoprecipitated by treating with antisera to murine C4 (23) or C3 adsorbed to protein A-Sepharose (Sigma) as described by Quaranta *et al.* (24). ^{35}S-Labeled translation products were fractionated on 7.5% $NaDodSO_4$/polyacrylamide gels (25) and visualized by impregnating the gel with EN^{3}HANCE (New England Nuclear) and autofluorography at −70°C.

cDNA Synthesis and Cloning. About 150 μg of size-fractionated (≥28 S) B10.WR poly(A)$^+$ RNA was resized on a sec-

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Abbreviations: C2, C3, and C4, second, third, and fourth components of complement, respectively; Slp, sex-limited protein; Ss, serum substance.

ond sucrose gradient. One-milliliter fractions were collected, precipitated twice with ethanol, and assayed for C4 mRNA by *in vitro* translation and immunoprecipitation. C4-positive fractions were pooled, giving about 8 μg of RNA; half of this was used to construct cDNA clones by standard methods (see, e.g., ref. 26). mRNA was denatured with 10 mM methylmercury hydroxide prior to initiation of first strand cDNA synthesis, which was carried out in the presence of 1 unit of RNase inhibitor (Biotec) per ml. Double-stranded cDNA was inserted at the *Pst* I site of pBR322 as described by Villa-Komaroff *et al.* (27) and the resulting recombinant plasmids were transformed into *Escherichia coli* strain MC1061 (28). Tetracycline-resistant, ampicillin-sensitive colonies were stored in microtiter plates essentially as described by Gergen *et al.* (29).

Differential Hybridization. Nitrocellulose filters (Millipore) that had been placed onto nutrient agar plates containing 20 μg of tetracycline per ml were inoculated in duplicate from the stocks by using a multi-pronged device. After overnight growth at 37°C, plasmid DNA was fixed to the filters by standard methods (26). Filters were probed pairwise with ^{32}P-labeled oligo(dT)-primed cDNA (30) from size-fractionated (≥28 S) B10.WR or B10.BR mRNA. Plasmid DNA from colonies showing differential hybridization to the two probes was analyzed by dot hybridization (31) to B10.WR or B10.BR mRNA (≥28 S), which had been labeled with ^{32}P by mild alkaline hydrolysis, followed by 5′-end labeling with polynucleotide kinase (32).

Hybrid-Selected Translation. Plasmid DNA was applied to 3-mm square nitrocellulose filters and hybridized to size-selected B10.WR poly(A)$^+$ RNA essentially as described (26). Only 2 μg of plasmid DNA was applied per filter, however. Hybridization was carried out for 90 min at 50°C with about 7 μl of solution per filter and 100 μg of size-selected B10.WR RNA per ml. RNA was released from the filters by boiling, extracted with phenol/chloroform, precipitated with ethanol, and assayed by *in vitro* translation.

DNA Sequence Analysis. The method of Maxam and Gilbert (33) was used.

RESULTS AND DISCUSSION

Detection of C4 mRNA by Cell-Free Translation. Murine C4 is biosynthesized primarily in the liver. To obtain C4 mRNA, we isolated poly(A)$^+$ RNA from livers of an inbred mouse, B10.WR, which exhibits high serum C4 levels (34). Our initial attempts to assay for C4 mRNA by immunoprecipitating the cell-free translation products of total B10.WR poly(A)$^+$ RNA were unsuccessful. Therefore, because we expected that C4 mRNA should be 6,000 bases in length or more, we fractionated B10.WR poly(A)$^+$ RNA by size on a sucrose gradient and translated the ≥28S fraction in the reticulocyte lysate system. We estimate from optical densities that this RNA fraction (≥28S mRNA) constitutes 10–20% of total poly(A)$^+$ RNA. Translation products were analyzed by immunoprecipitation with anti-C4 serum and, as a control, with antiserum against murine third complement component (C3). As shown in Fig. 1, the primary translation products recognized by C3 and C4 antisera have gel mobilities corresponding to M_rs of 185,000 and 190,000, respectively; we assume that they represent the full-length translation products of C3 and C4 mRNA. The remaining bands are nonspecific (in particular, the bands at about M_r 50,000, which are present even in the absence of exogenous RNA) or are assumed to be prematurely truncated products of C3 and C4 mRNA translation. These results are in close agreement with those of Odink *et al.* (35), who first characterized murine C3 and C4 mRNA and their cell-free translation products, and with the results of analogous studies of human C3 and C4 by Woods *et al.* (36). Cell-free translation/immunoprecipitation of <28S poly(A)$^+$ RNA showed no C3 or C4 products. Therefore, because the ≥28S fraction constitutes about 10–20% (by mass) of total poly(A)$^+$ RNA, we estimate that fractionation results in at least a 5- to 10-fold enrichment for C4 mRNA.

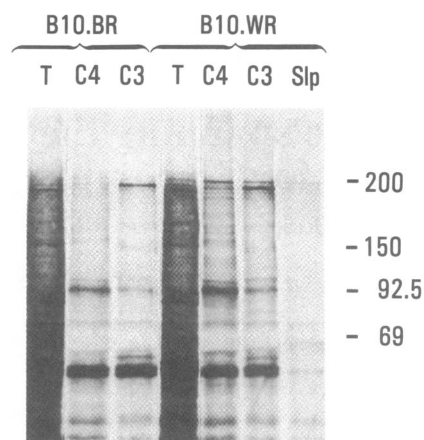

FIG. 1. Autoradiogram of a 7.5% $NaDodSO_4$/polyacrylamide gel showing the products of cell-free translation of size-selected (≥28 S) poly(A)$^+$ mouse liver RNA from the low-serum C4 strain, B10.BR, and the congeneic high-serum C4 strain, B10.WR. One microliter of total translation reaction (T) and the products of immunoprecipitating 10 μl of the reaction mix with antisera against C4, C3, and Slp were run on the gel. The B10.WR-T lane was exposed for 2 days; the others, for 8 days. Molecular weights are shown as $M_r \times 10^{-3}$.

The efficiency of immunoprecipitating C3 and C4 cell-free translation products is quite low in our studies; this probably accounts for our inability to detect these translation products with total poly(A)$^+$ RNA. In Fig. 1, the lane marked B10.WR-T represents 1 μl of total translation mix autoradiographed for 2 days, whereas the lanes marked B10.WR-C4, -C3, and -Slp represent the products of immunoprecipitating 10 μl of translation mix autoradiographed for 8 days. Assuming that all of the comigrating material in the total lane is C3 or C4, we estimate that immunoprecipitation is only 5–10% efficient. Attempts to enhance the antigenicity of C4 by translating in the presence of dog pancreas microsomes (37) were unsuccessful (data not shown).

Immunoprecipitation with alloantisera against Slp showed a light band comigrating with C4, but about 1/10th as intense (Fig. 1). If this band does represent the translation product of Slp mRNA, its intensity is 1/5th that expected because the ratio of C4 to Slp protein in B10.WR serum is about 2:1 (unpublished data). This discrepancy might be due to experimental errors, to disproportionately inefficient immunoprecipitation of Slp cell-free translation products with Slp alloantiserum, to a lack of correspondence between liver mRNA levels and protein levels in serum, or to a combination of all three. We cannot at this time distinguish between these possibilities.

Strain-Dependent Differences in Serum C4 Levels Are Reflected in Levels of C4 mRNA. Ten- to 20-fold differences in serum C4 levels exist among inbred strains of mice (reviewed in 34). The results of translating ≥28S mRNA from the high C4 strain, B10.WR (S^{w7}), and from the congeneic low C4 strain, B10.BR (S^k), are compared in Fig. 1. B10.WR carries a recombinant *H-2* haplotype that apparently resulted from a crossover between the wild-derived $H\text{-}2^{w7}$ haplotype and the $H\text{-}2^k$ haplotype of B10.BR; backcrosses to the latter strain were made to establish the B10.WR congeneic strain (38). Because the crossover point lies between *S* and *D*, we expect the C3 locus, which maps to the right of D, to be identical for both B10.BR and B10.WR mice (39). Fig. 1 shows, as expected, that mRNA from

the two strains directs the synthesis of comparable amounts of C3. The levels of C4 synthesized are quite different, however; C4 synthesis is much greater with B10.WR than with B10.BR. This difference is especially clear in the "total" lanes, where the ratio of C3 to C4 products can be seen. The result parallels the ≈20-fold greater amount of C4 found in B10.WR serum relative to B10.BR (34); it implies that the difference in C4 levels between these strains is due, at least in part, to differences in steady-state levels of C4 mRNA. We cannot exclude the possibility of strain-dependent differences in cell-free translation of C4 mRNA or differences in immunoprecipitation due to C4 polymorphisms (the antiserum used was raised against C4 from B10.WR mice). However, the observations in cloning C4 cDNA that follow confirm the lower level of C4 mRNA in B10.BR.

Cloning C4 cDNA. C4 mRNA is very large and is expected to constitute <0.2% of the total message population (40). Therefore, to simplify identification of C4 clones, a preparation of B10.WR liver mRNA was fractionated twice on sucrose gradients, giving a fraction we estimate to be 25- to 100-fold enriched for C4 mRNA. This material was used to construct cDNA clones by standard methods. About 4 μg of mRNA yielded 1,000 recombinant clones. Because our earlier results indicated a substantially higher level of C4 mRNA in B10.WR than in the congeneic B10.BR strain, we examined our clones by comparing the level of hybridization to mRNA from these two strains. We expected that any differences in the intensity of hybridization would be due only to differences in levels of C4 mRNA or mRNA transcripts under the control of other *H*-2K proximal genes (39). In an initial screening, 200 clones were analyzed by comparative hybridization to ≥28S mRNA from B10.WR and B10.BR mice. We used size-fractionated mRNA here (5- to 10-fold enriched for C4 mRNA) to increase the proportion of C4 sequences in the probes and hence to increase the specific activity. Reconstitution experiments with purified probes suggest that a specific sequence must constitute >0.05% of the probe to be seen above the background in colony blots (30).

Our results are shown in Fig. 2. Most of the colonies show some level of hybridization to the two probes, with hybridization essentially identical for 90% of the clones. Of the remaining 20 colonies, 15 showed greater hybridization to the B10.WR probe. Although the clones were constructed from B10.WR RNA, 5 showed greater hybridization to the B10.BR probe. Reanalysis of the 20 clones by dot hybridization (31) affirmed differential hybridization for 9 of the 15 clones hybridizing preferentially to the B10.WR probe and 1 of the 5 clones hybridizing more strongly to B10.BR. The 9 clones showing greater hybridization to the B10.WR probe in both tests were analyzed for the ability to hybridize to C4 mRNA by hybrid-selected translation experiments. Three of the nine clones selected C4 message (data not shown). In Fig. 2 they correspond to the clones in the middle panels, row 6, column 5 (clone A), and the clones in the bottom panels, row 1, column 6 (clone B), and row 3, column 6 (clone C). Restriction enzyme analysis revealed that clones B and C are identical. Thus, of 200 clones, two unique plasmids, designated pMLC4/w7-2 (clone A) and pMLC4/w7-3 (clone B), were isolated. The remaining clones showing differential hybridization have not been analyzed further. From the frequency of C4 clones, we estimate that C4 mRNA constitutes 0.01–0.04% of the total mouse liver mRNA species.

DNA sequence analysis of pMLC4/w7-2 and pMLC4/w7-3 revealed that these plasmids carry cDNA inserts 458 and 533 base pairs long, respectively. Translating the nucleic acid sequence into protein sequence, we found with each plasmid that only one of the six possible reading frames encodes a contiguous amino acid sequence across the entire insert. We assume that the amino acid sequences encoded in these two reading frames represent segments of the pro-C4 molecule. Fig. 3 shows the DNA sequence of the insert in pMLC4/w7-2 and the 152-residue-long amino acid sequence encoded by the only open reading frame spanning the insert. A distinctive feature of the amino acid sequence is the arginine quartet translated from bases 254–266 in the nucleotide sequence; it is reminiscent of the arginine quartet at the β–α subunit junction in murine pro-C3 determined by Domdey *et al.* (43). Given the similarity between C3 and C4, this suggests that the arginines represent a processing site. If this is the case, maturation of C3 and C4 probably involves the same or very similar proteases. Unlike pro-C3, which is cleaved once, at the β–α junction, to yield the C3 dimer, pro-C4 is cleaved twice: at the β–α junction and at the α–γ junction. If the four arginines in our sequence signal a cleavage site, which site is it? Fig. 3 shows that, of the 21 amino acids immediately following the last arginine in the quartet, 18 match the available NH_2-terminal sequence of the human C4 γ chain (15, 41, 42). Only very limited amino acid sequence data exist for the murine C4 γ chain: microsequence analysis of radiolabeled protein has placed a lysine at residue 4 and valines at residues 5, 13, and 17 (44). These assignments agree completely with our results, including the absence of valine at residue 6. We conclude that the arginine quartet in our putative amino acid sequence *is* a cleavage site and that it marks the junction between the α and γ subunits of murine pro-C4.

The level of homology between the human and putative mouse γ-chain sequences (85%) is quite high. Domdey *et al.* (43) found only 68% homology between mouse and human C3a sequences and 85% homology between mouse and human sequences in the vicinity of the thiolester site of C3, a region which is central to C3 function and thus highly conserved; this suggests that the NH_2 terminus of the γ chain might also play an important role in C4 structure or function, or both.

An additional piece of evidence that pMLC4/w7-2 carries a sequence that includes the α–γ junction of pro-C4 lies in the

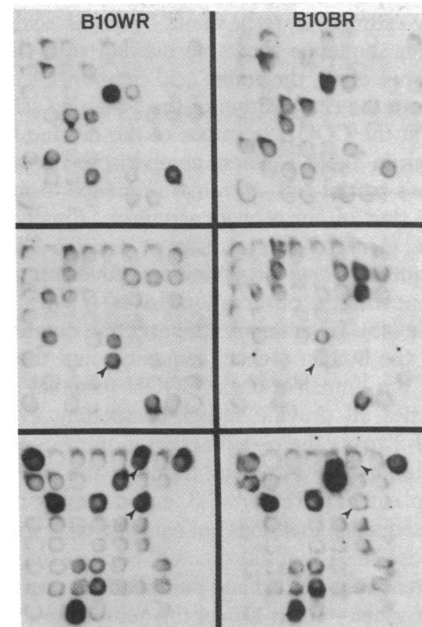

FIG. 2. Autoradiogram of colony clots showing differential hybridization of B10.WR liver cDNA clones to ^{32}P-labeled cDNA from B10.WR and B10.BR liver mRNA. Clones were applied to nitrocellulose filters in duplicate (rows) and probed (columns) with labeled cDNA. The arrowheads mark the colonies showing hybridization to C4 mRNA in the hybrid-selected translation assay. The clone in the middle panel (pMLC4/w7-2) carries a cDNA insert spanning the α–γ junction in pro-C4.

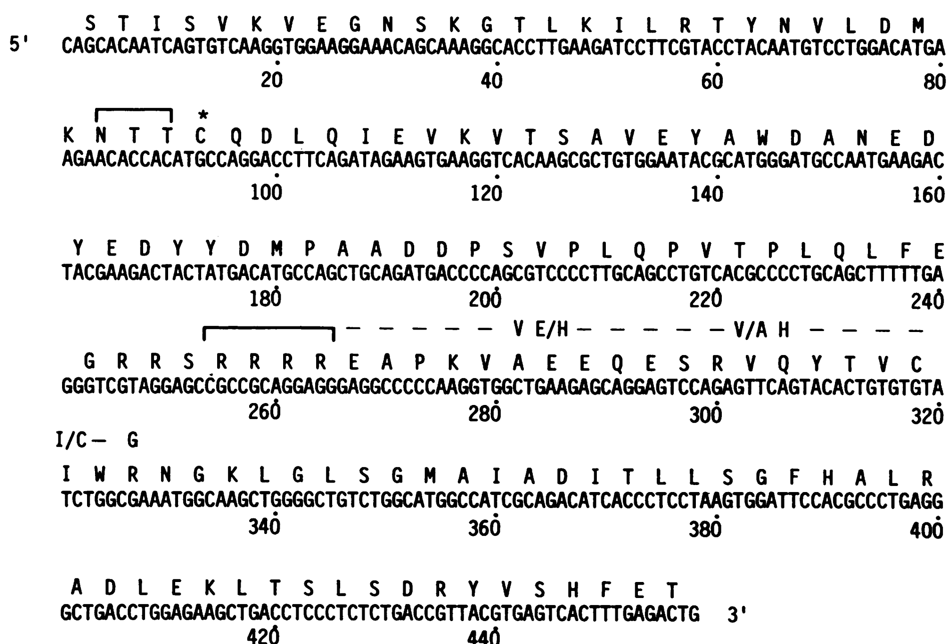

FIG. 3. Nucleotide sequence of the cDNA insert in pMLC4/w7-2. The sequence has been translated into protein sequence for the only open reading frame spanning the insert. The brackets mark a possible glycosylation site at the α-chain COOH terminus and the arginine tetramer, which we propose signals the α–γ cleavage site in pro-C4. The C4 γ chain starts immediately after the four arginines. Here, the human C4 γ-chain sequence is given above our protein sequence. Dashes indicate amino acid identity. Dual assignments indicate sites of disagreement between the two reported human γ-chain sequences (15, 41, 42).

putative α-chain sequence of PMLC4/w7-2. Assuming that the arginine quartet is totally excised in processing, as is the case with the β–α junction of pro-C3 (43), then the preceding 84 amino acids lie at the COOH terminus of the C4 α chain. No sequence information is available in this region for any species. However, Press and Gagnon (45) have determined the partial NH_2-terminal sequence and amino acid composition of a 12,000-dalton glycopeptide (α_4) from this region of the human C4 α chain. They estimate α_4 to be about 86 amino acids in length. Although our sequence shows no overlap with the NH_2-terminal sequence of α_4, the amino acid composition of α_4 agrees very well with the composition of the 84-amino acid sequence we infer is at the COOH terminus of the murine C4 α chain; this is shown in Table 1. These comparisons imply that Press and Gagnon's partial NH_2-terminal sequence terminates very close to the start of our α-chain sequence. Finally, the glycosylation site, Asn-Thr-Thr (starting at position 83 in the nucleotide sequence), and the cysteine residue just downstream are consistent with the observation that α_4 is a disulfide-linked glycopeptide (45). In summary, the arginine quartet, the close match with the human γ-chain sequence, and the correspondence with the human α-chain COOH-terminal composition and properties all provide rather compelling evidence that pMLC4/w7-2 spans the α–γ junction in murine pro-C4.

The single long open reading frame in the cDNA insert of the second plasmid, pMLC4/w7-3, encodes a 177-residue-long amino acid sequence that does not coincide with any published C4 sequences. This is not unexpected, because only human C4 has been extensively studied and published sequences total only about 300 residues—about 15% of the number anticipated from the size of C4. Additional protein or cDNA sequence information will be required to place the sequence encoded by pMLC4/w7-3.

Differential hybridization of pMLC4/w7-2 and pMLC4/w7-3 to mRNA from B10.WR and B10.BR confirms the difference in steady-state levels of C4 mRNA between these inbred strains, which we inferred from cell-free translation experiments. Moreover, differential hybridization provided a fast and simple primary screening method to decrease to 1/20th the number of clones eventually analyzed by the more specific hybridization-selected cell-free translation. Given the difficulties inherent in the latter method, in particular with an exceptionally long mRNA, differential hybridization was almost essential in these studies.

Table 1. Comparison of the amino acid composition of α_4 and the putative α-subunit COOH-terminal region in pMLC4/w7-2

Amino acid residue	α_4 (Human)*	α COOH terminus (mouse)†
Asx	11	8 + 4‡
Thr	5	7
Ser	4	6
Glx	13	6 + 4‡
Pro	2	5
Gly	9	3 (6)
Ala	4	5 (6)
Val	7	8
Cys	1	1
Met	2	2
Ile	3	3
Leu	8	7 (10)
Tyr	4	5
Phe	2	1 (2)
His	2	0
Lys	6	5 (6)
Arg	3	3
Trp	—	1
Total	86	84 (93)

* α_4 NH_2-terminal sequence (45) Gly-Gly-Phe-Lys-AA-Leu-Ala-Leu-AA-Leu-Gly (AA, amino acid residue).

† Numbers in parentheses show the effect of adding the amino acids at the NH_2 terminus of α_4 to the sequence from pMLC4/w7-2.

‡ Acid + amide.

Because C4 and Slp are very similar structurally, we expect their cDNAs to be indistinguishable at the present level of analysis. Therefore, we cannot exclude the interesting possibility that one or both of our clones carry Slp rather than C4 cDNA sequences. As discussed earlier, the likelihood that one of these clones carries Slp cDNA ranges from 30% (from relative levels of C4 and Slp in B10.WR serum) to 10% (based on the present cell-free translation results). The isolation and sequence analysis of additional cDNA clones and more detailed hybridization studies should resolve this question.

Is the COOH Terminus of the C4 α Chain Proteolytically Cleaved in Plasma? Recent work has demonstrated that the α chain of both human and murine C4 undergoes some form of postsynthetic processing in plasma (46, 47): upon secretion, the α chain rapidly matures ($t_{1/2} \approx 1$ hr) to a form showing an increased mobility on $NaDodSO_4$/polyacrylamide gels consistent with a decrease in size of about 4,000 daltons. Because both secreted and plasma α chains appear identical at their NH_2 termini, processing apparently involves the loss of about 40 amino acids at the COOH terminus. However, because the previously described peptide, α_4, was isolated from the plasma form of human C4, the close match in amino acid compositions between α_4 and our proposed murine α-chain COOH-terminal sequence (Table 1) argues against the loss of as many as 40 terminal amino acids. The available data do not indicate whether this apparent inconsistency is due to a fortuitous but false match in the amino acid compositions or to the sometimes unpredictable effects of small structural modifications on protein mobilities on $NaDodSO_4$/polyacrylamide gels. For example, loss of the highly positively charged trimer at the COOH terminus of our putative α-chain sequence or some other chemical modification might disproportionately alter the gel mobility of the α chain. More sequence information either at the DNA or protein level will resolve this question.

We thank M. Wilson, D. Levy, G. Fey, S. Riley, V. Quaranta, P. Branks, G. Sutcliffe, and F.-T. Liu for help with techniques and many valuable suggestions, G. Sutcliffe, M. Wilson, and J. P. Atkinson for comments on the manuscript, and S. Baldwin for preparing the manuscript. This work was supported by U.S. Public Health Service Grants GM29831, AI12734, and AI15353 and by an American Cancer Society Junior Faculty Research Award (R.T.O.). This is publication no. 3020-IMM from the Research Institute of Scripps Clinic.

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