Brief Definitive Report

The Reduced Expression of 6Ckine in the *plt* Mouse Results from the Deletion of One of Two 6Ckine Genes

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Summary

6Ckine is an unusual chemokine capable of attracting naive T lymphocytes in vitro. It has been recently reported that lack of 6Ckine expression in lymphoid organs is a prominent characteristic of mice homozygous for the paucity of lymph node T cell (*plt*) mutation. These mice show reduced numbers of T cells in lymph nodes, Peyer's patches, and the white pulp of the spleen. The genetic reason for the lack of 6Ckine expression in the *plt* mouse, however, has remained unknown. Here we demonstrate that mouse 6Ckine is encoded by two genes, one of which is expressed in lymphoid organs and is deleted in *plt* mice. A second 6Ckine gene is intact and expressed in the *plt* mouse.

Key words: chemokines • cell trafficking • leukocyte chemotaxis • T lymphocytes • mutation

Also reported as secondary lymphoid organ chemokine (1), Exodus-2 (2), and thymus-derived chemotactic agent 4 (3), 6Ckine is a unique CC chemokine containing six cysteines (4). Four of these cysteines occur in the expected conserved positions, and two additional cysteines are arrayed in a highly charged COOH-terminal domain unique to 6Ckine (4). In humans as well as mice, expression of 6Ckine mRNA is highest in lymphoid tissues, particularly LN and spleen, but 6Ckine is also expressed at lower levels in some nonlymphoid tissues (1, 2, 4, 5). Expression of 6Ckine in LNs and Peyer's patches (PPs) has been more precisely localized by in situ hybridization to high endothelial venules (6). Expression of 6Ckine also occurs in the T cell areas of spleen, LN, and PP (5–7), as well as in thymic stromal cells (3). Expression of 6Ckine in other tissues, such as liver and small intestine, has been localized to the lymphatics (6).

In vitro, 6Ckine is reported to attract T cells, B cells, thymocytes, dendritic cells, and mesangial cells but not neutrophils or monocytes (1–9). It has been reported that 6Ckine preferentially attracts naive T cells over memory T cells (6), although this preference has not been universally observed (2, 7). Several investigators have also reported that activated T cells migrate more vigorously toward 6Ckine (4, 5, 7). In addition to its chemotactic activities, 6Ckine promotes adhesion of lymphocytes under shear flow conditions (6, 10, 11). These reports also suggest that the adhesion-promoting effects of 6Ckine are more pronounced for naive versus memory T cells. Taken together, these observations suggest that 6Ckine is involved in homing of lymphocytes or dendritic cells to secondary lymphoid organs. One prediction of this hypothesis is that 6Ckine null mice would show defects in homing of these cells to LNs, leading to a reduction in LN cellularity. Interestingly, a mutant mouse displaying just such a phenotype had been previously described by Nakano et al. (12–14) as the DDD/1 mouse.

The DDD/1 mouse carries an autosomal recessive mutation designated paucity of lymph node T cells (*plt*) and shows dramatically reduced numbers of T cells in the peripheral LN, PP, and spleen, whereas memory T cell numbers are unchanged (14). Interestingly, B cell numbers in the lymphoid organs of these mice are also relatively normal. Accumulation of neutrophils and macrophages in response to intraperitoneal injection of thioglycolate also appears normal in *plt* mice. Recent characterization of the *plt* phenotype by Gunn et al. has shown that these mice lack 6Ckine expression in lymphoid organs (15). This report also found that dendritic cell numbers were reduced in LNs. However, no significant alterations in the 6Ckine gene were found by these investigators, and thus the precise nature of the genetic defect in the *plt* mouse has remained unknown.

Our interest in the normal function of 6Ckine led us to begin work on a targeted disruption of the 6Ckine gene. In the process, we discovered that 6Ckine is encoded by two genes in the mouse genome. The predicted products of these two genes are nearly identical, differing by only a single amino acid. Furthermore, we demonstrate that one of the two 6Ckine genes is deleted in the *plt* mouse, leading

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to a loss of 6Ckine expression in lymphoid organs, whereas expression in various nonlymphoid organs, presumably arising from the remaining gene, is maintained.

Materials and Methods

Isolation and Characterization of the Genomic Copies of the 6Ckine Gene. Two bacterial artificial chromosome (BAC) clones containing genomic copies of the mouse 6Ckine gene were identified in a mouse 129/sv embryonic stem cell genomic library (Genome Systems, Inc.) using PCR primers corresponding to the mouse 6Ckine cDNA: GV100 (5′-CTG CAA GAG AAC TGA ACA GAC-3′) and GV105 (5′-CTT CTG ACT CTC TAG GTC TAC-3′). Several overlapping fragments containing the 6Ckine gene were identified by Southern blot analysis of BAC plasmid DNA using a 275-bp PCR-generated probe (GV100/GV105) labeled with [^{32}P]dCTP (Amersham Corp.; 3,000 Ci/mmol) by random priming (Megaprime DNA Labeling System; Amersham Corp.). They were subcloned into pBluescript (Stratagene Inc.) and mapped by restriction digest. 6Ckine-containing SacI fragments (7.5 kb) from each of the two BAC clones were sequenced using an Applied Biosystems 377 sequencer (Applied Biosystems, Inc.).

PCR Analysis of the 6Ckine Locus. A pair of PCR primers GV104 (5′-GTA GAC CTA GAG AGT CAG AAG-3′) and GV125 (5′-CGC GGA TCC TTG GAG GAG GAA CCA CAG T-3′), shown in Fig. 2, were used to amplify 1.35- and 1.2-kb fragments that included part of the 3′ untranslated region (UTR) and ~1 kb downstream of the gene. PCR conditions were: 94°C for 2 min; 25 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min; and 72°C for 5 min. Both fragments were subcloned into pCR2.1 TA cloning vector (Invitrogen Corp.) and sequenced. Another pair of PCR primers, GV95 (5′-ATG GCT CAG ATG ATG ACT CT-3′) and GV105 (5′-CTT CTG ACT CTC TAG GTC TAC-3′) (see Fig. 2) were used to amplify the 5′ coding region and part of the 3′ UTR of the 6Ckine gene (899 bp) from wild-type and BALB/c-*plt* (10th backcrossed generation) tail DNA. These fragments were also subcloned into pCR2.1 TA cloning vector (Invitrogen Corp.) and sequenced.

Southern Blot Analysis of 6Ckine Locus. Tail DNA was isolated from 129/sv (The Jackson Laboratory) and BALB/c-*plt* mice. EcoRI and HindIII (New England Biolabs Inc.)–digested mouse tail DNA and BAC DNA were denatured and blotted onto Duralon membrane (Stratagene Inc.) and hybridized with probes A and B (see Fig. 3).

RNA Analysis. Total RNA was extracted from different tissues of *plt* and BALB/c mice using Ultraspec RNA Reagent (Biotecx Labs., Inc.), and 20 μg/lane was electrophoresed in a 1% agarose gel. The RNA was blotted onto Duralon nylon membrane (Stratagene Inc.). A 6Ckine probe was labeled with [^{32}P]dCTP (Amersham Corp.; 3,000 Ci/mmol) by random priming (Megaprime DNA Labeling System; Amersham Corp.). Hybridization was carried in QuikHyb hybridization solution (Stratagene Inc.).

Results and Discussion

Mouse 6Ckine Is Encoded by Two Separate Genes. As part of an effort to generate 6Ckine null mice, a genomic library generated from 129/sv embryonic stem cells was screened by PCR (see Materials and Methods). Two BAC clones were identified, which we designated 6CKBAC1 and 6CKBAC2. Southern blot analysis of EcoRI-digested DNA from each BAC clone revealed two distinct banding patterns (Fig. 1 A). 6CKBAC1 contained bands of 6.0 and 1.35 kb, whereas 6CKBAC2 contained two bands of 3.0 and 1.2 kb. Southern blot analysis of 129/sv mouse genomic DNA showed that all four bands were present. These results immediately suggested the possibility of two distinct 6Ckine genes in the mouse genome. Interestingly, hybridization of *plt* mouse genomic DNA showed only bands corresponding to those found in 6CKBAC1 (Fig. 1 A), suggesting the possibility that one of the two 6Ckine genes in the *plt* mouse is altered.

To further investigate this possibility and to determine if this was a strain-specific observation, two primers (GV104 and GV125; Fig. 2) amplifying a segment of the 3′ UTR of the 6Ckine gene were used to amplify genomic DNA from various mouse strains as well as the two BAC clones. DNA from three separate mouse strains, BALB/c, 129/sv, and C57BL/6, all demonstrated two bands of 1.35 and 1.2 kb (Fig. 1 B). 6CKBAC1 showed only a 1.35-kb band and 6CKBAC2 only a 1.2-kb band (Fig. 1 B). These data suggest that wild-type mice have two 6Ckine genes, one of which is represented on 6CKBAC1 and the other on 6CKBAC2. Interestingly, PCR amplification of *plt* mouse genomic DNA showed only a single 1.35-kb band identical to the size of the band amplified from 6CKBAC1 (Fig. 1 B). This result again suggested that the *plt* mouse has an alteration in one of the two 6Ckine genes.

To conclusively demonstrate that the 6Ckine gene on 6CKBAC1 was indeed different from that residing on 6CKBAC2, SacI fragments (7.5 kb) were isolated from each BAC, subcloned, and completely sequenced. Analysis of the sequence data clearly showed that each BAC contained

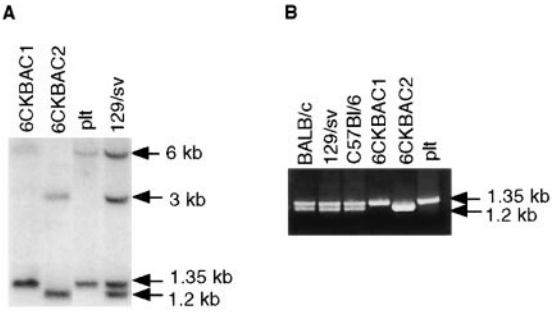

Figure 1. PCR and Southern blot analysis of the 6Ckine gene locus reveals the existence of two 6Ckine genes, only one of which is detected in the *plt* mouse. (A) EcoRI-digested DNA from 6CKBAC1, 6CKBAC2, *plt*, and 129/sv mice was analyzed by Southern blot analysis using a ^{32}P-labeled NheI/BstXI fragment (271 bp) as a probe (probe B, Fig. 3 C). The probe specifically hybridized to 6- and 1.35-kb fragments in 6CKBAC1 DNA, 3- and 1.2-kb fragments in 6CKBAC2, 6- and 1.35-kb fragments in *plt* mouse genomic DNA, and all four fragments in 129/sv genomic DNA. (B) PCR analysis: PCR primers GV104/GV125 (see Fig. 2) were used to amplify a segment of the 3′ region of the 6Ckine gene. The resulting PCR products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide. Two bands of 1.35 and 1.2 kb were detected in a PCR reaction from genomic DNA from several mouse strains (as indicated). Single bands of 1.35 or 1.2 kb were detected from 6CKBAC1 and 6CKBAC2 DNA, respectively, whereas a single band of 1.35 kb was amplified from the *plt* mouse genomic DNA.

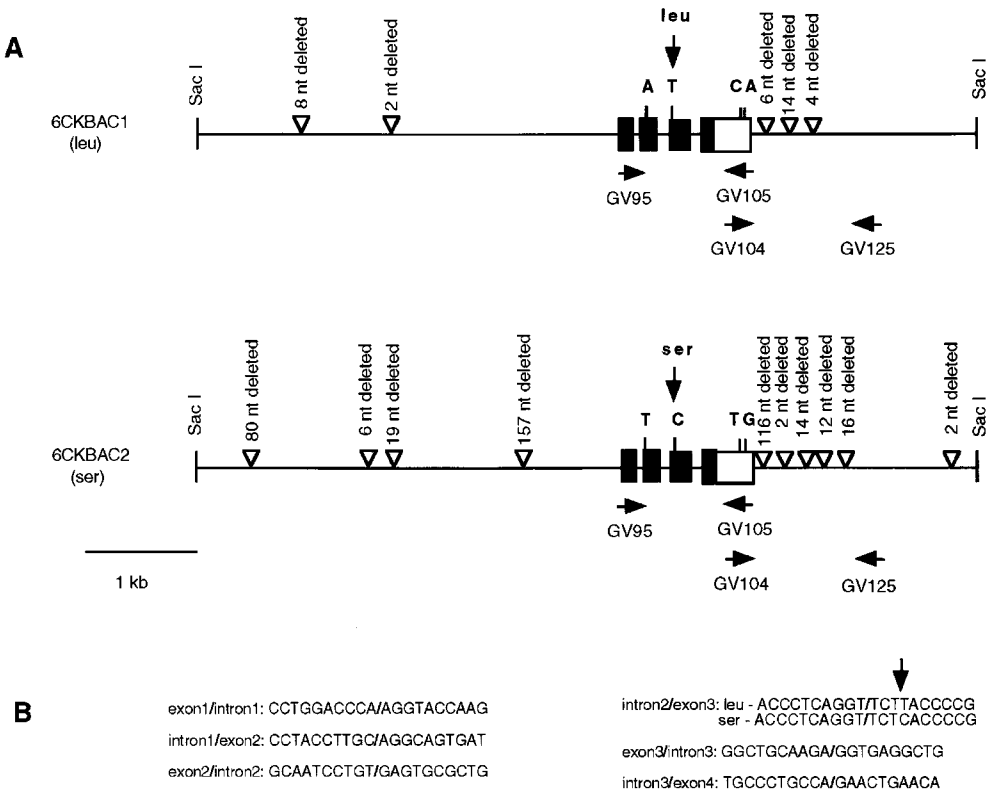

Figure 2. (A) The 6Ckine genes found on the two BACs are distinct. A schematic of the sequence of SacI fragments (7.5 kb) from 6CKBAC1 (leu) and 6CKBAC2 (ser) is shown. Black and white boxes represent 6Ckine exons. Open triangles indicate larger deletions. Single base differences outside of the exons are not shown. The four nucleotide differences identified in coding regions (black boxes) and noncoding regions (white box) of the two 6Ckine genes are shown. Vertical arrows identify the amino acid difference at position 65. Horizontal arrows indicate the positions of the PCR primers used for analysis of 6Ckine gene locus. nt, nucleotide. (B) Sequences of the intron–exon boundaries are identical between the two copies with the exception of the intron2–exon3 junction. The 6Ckine-ser sequence is added to the intron2–exon3 boundary sequence to illustrate the single nucleotide difference (arrow). These sequence data are available from EMBL/GenBank/DDBJ under accession numbers AF171085 and AF171086 for 6Ckine-leu and 6Ckine-ser, respectively.

a distinct 6Ckine gene (Fig. 2). Furthermore, although the overall genomic organization of the two 6Ckine genes was similar, consisting of four exons and three introns (Fig. 2), there were a number of sequence differences. These differences included numerous single base changes as well as various small (<200 bp) deletions (Fig. 2). Within the coding regions of the two genes, only two single nucleotide differences were found, one of which resulted in an amino acid difference at position 65. 6CKBAC1 encodes a leucine at this position, whereas 6CKBAC2 encodes a serine. A series of deletions/insertions in a region of the gene downstream from the polyadenylation signal results in a net difference of 136 bp between the two genes that accounts for the observed difference in the size of the PCR products amplified by GV104/GV125 primers (Fig. 1 B). Sequencing of these two PCR fragments amplified from wild-type genomic DNA shows that the sequence of each exactly matches that of the appropriate BAC, further proving that 6Ckine is encoded by two genes and eliminating the possibility that our previous observations were the result of a BAC cloning artifact. Together, these data clearly demonstrate that there are two 6Ckine genes in mice and that each BAC carries a unique 6Ckine gene. Based on the differences in the amino acid sequence of 6Ckine derived from each gene, we designate the two chemokines 6Ckine-leu (encoded on 6CKBAC1) and 6Ckine-ser (encoded on 6CKBAC2).

The plt Mouse Has Deleted One of Two 6Ckine Genes. The initial analysis of the *plt* mouse DNA (Fig. 1) suggested that these mice might have an alteration in one of the two 6Ckine genes. To confirm this hypothesis and to characterize these two genes in *plt* mice, a pair of primers (GV95/GV105) was used to amplify an 800-bp fragment of both genes from wild-type and *plt* genomic DNA. PCR products were then subcloned and sequenced. DNA amplified from a wild-type mouse would be predicted to consist of sequences encoding both forms of 6Ckine, and indeed, 14/24 subclones derived from the PCR reaction encoded serine at position 65, whereas 10/24 subclones encoded leucine at this position. In contrast, DNA amplified from the *plt* mouse showed 12/12 subclones encoding leucine at position 65. These data confirm that 6Ckine is encoded by two separate genes in the wild-type mouse genome and suggest that the *plt* mouse is likely to contain mutations in the serine form of 6Ckine. Furthermore, the fact that two separate primer pairs failed to amplify the 6Ckine-ser gene suggested that the *plt* mouse has a deletion in this gene.

To demonstrate that the *plt* mouse has indeed deleted one 6Ckine gene, two DNA probes corresponding to the 5′ end of the gene (probe A, Fig. 3 C) and the 3′ end of the gene (probe B, Fig. 3 C) were hybridized to DNA from 129/sv and *plt* mice as well as 6CKBAC1 and 6CKBAC2. Hybridization with probe A revealed two bands of the expected sizes in EcoRI-digested DNA from 129/sv mice (Fig. 3 A). Only one band, corresponding to the leucine form

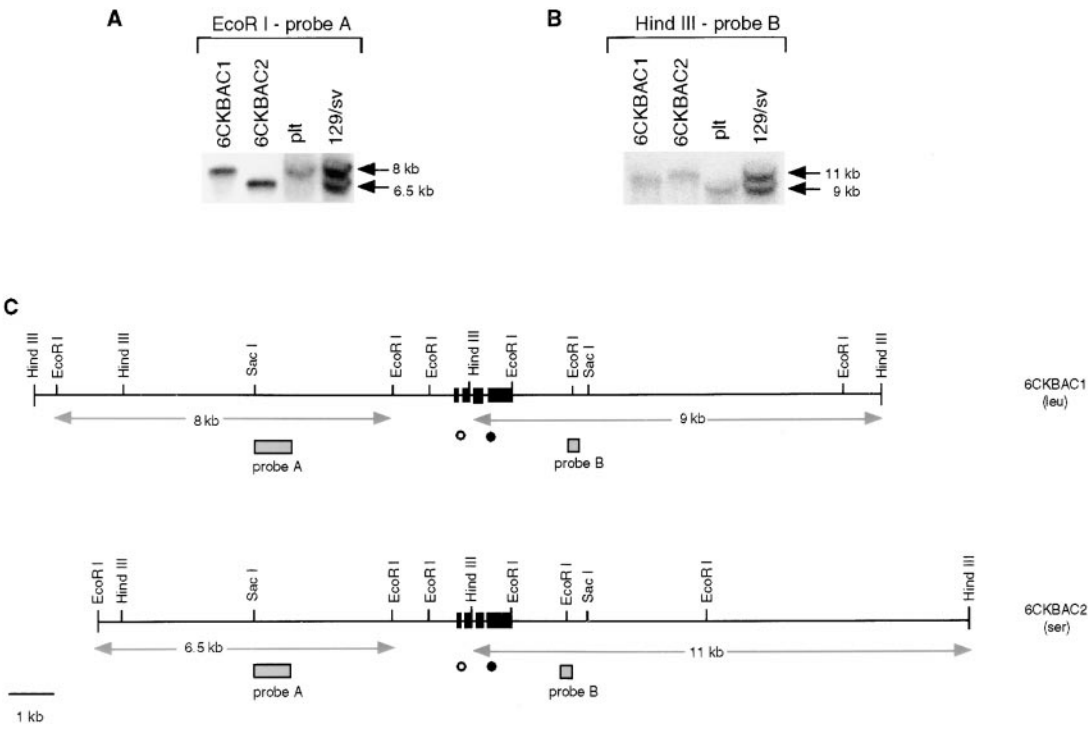

Figure 3. Southern blot analysis of 6Ckine genomic loci suggests that the 6Ckine-ser gene of the *plt* mouse contains significant deletions. (A) EcoRI-digested genomic DNA from 6CKBAC1 and 6CKBAC2 and genomic DNA from 129/sv and *plt* mice were probed with a 0.8-kb SacI/NheI fragment (probe A, indicated). An 8-kb fragment was recognized in 6CKBAC1, and a 6.5-kb fragment was recognized in 6CKBAC2. The *plt* mouse genomic DNA contained only an 8-kb fragment DNA, whereas both 8- and 6.5-kb fragments hybridized in 129/sv DNA. (B) HindIII-digested DNA from 6CKBAC1 and 6CKBAC2 and genomic DNA from 129/sv and *plt* mice was probed with a 271-bp NheI/BstXI fragment (probe B, indicated). A 9-kb fragment hybridized in 6CKBAC1 and an 11-kb fragment hybridized in 6CKBAC2 DNA. Only a 9-kb fragment was recognized in *plt* genomic DNA, whereas both 9- and 11-kb fragments were present in 129/sv DNA. (C) Schematic of the 6Ckine genomic locus, with positions of initiation and stop codons indicated by ○ and ●, respectively. The positions of the hybridization probes are indicated by shaded boxes, and the sizes of the fragments recognized by these probes relative to each BAC are indicated.

of 6Ckine, was detected in *plt* mice. A band of the same size was also detected in 6CKBAC1 (encoding 6Ckine-leu) but not in 6CKBAC2 (encoding 6Ckine-ser). Similarly, hybridization with probe B showed a pattern of bands in HindIII-digested 129/sv DNA consistent with the presence of both genes, whereas *plt* mice showed only bands of the same size as those present in 6CKBAC1. The failure of two independent probes, encompassing at least 7 kb of the 6Ckine gene, to hybridize to *plt* mouse DNA corresponding to 6Ckine-ser gene shows that the *plt* mouse has a deletion in the 6Ckine-ser gene and that this deletion likely includes all of the 6Ckine-ser coding region.

The plt Mouse Expresses 6Ckine in Nonlymphoid Organs. Our data demonstrates that the *plt* mouse has deleted the 6Ckine-ser gene but that it retains an intact 6Ckine-leu gene. A previous report has shown that the *plt* mouse lacks 6Ckine expression in lymphoid tissue, but expression in other tissue types was not reported (15). As the 6Ckine-leu gene is present in the *plt* mouse, we thought it possible that these mice might express 6Ckine in nonlymphoid tissue. To examine this possibility, we conducted Northern blot analysis on a variety of both wild-type (BALB/c) and *plt* mouse organs. Consistent with our previously reported findings (4), normal mice showed expression of 6Ckine in both lymphoid and nonlymphoid organs (Fig. 4 A). As expected, *plt* mice were found to lack detectable expression of 6Ckine mRNA in lymphoid organs (Fig. 4 B). Expression of 6Ckine was observed, however, in a number of nonlymphoid organs of the *plt* mouse (Fig. 4 B). As the 6Ckine-ser gene is deleted in these mice, the observed 6Ckine message likely derives from expression of the 6Ckine-leu gene.

6Ckine/secondary lymphoid organ chemokine has been demonstrated in vitro to be important for lymphocyte adhesion and migration. Recent findings have also suggested a role for this chemokine in dendritic cell migration (8, 9). The lack of 6Ckine expression in the lymphoid organs of the *plt* mouse, along with the reported phenotype of these mice, suggests that at least some of these observations are also true in vivo. The conclusions drawn from the *plt* mouse regarding 6Ckine are confounded by the fact that the precise nature of the mutation in these mice remains unknown. We have presented data here that demonstrates that murine 6Ckine is, in fact, encoded by two separate genes and that one of these is deleted in the *plt* mouse. The two 6Ckine genes are nearly identical in the sequences of their open reading frames and show only one amino acid difference at position 65.

Analysis of the public expressed sequence tag (EST) database shows that cDNAs for both forms of mouse 6Ckine

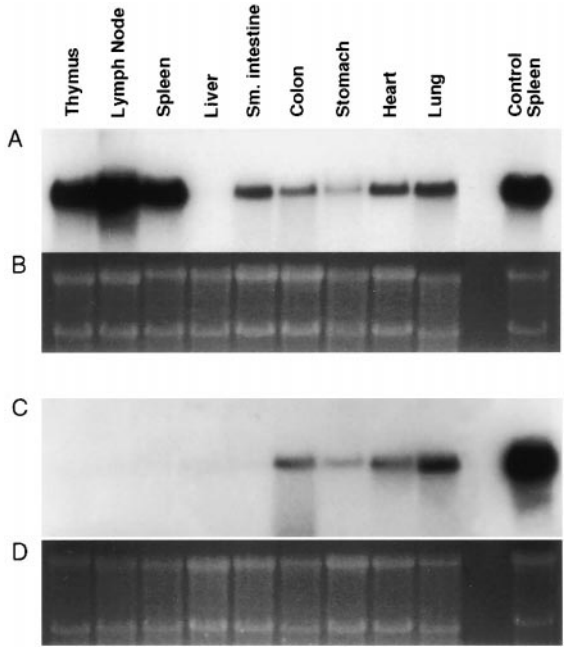

Figure 4. 6Ckine is expressed in both wild-type and *plt* mice. Total RNA (20 μg/lane) from either BALB/c (A) or *plt* (C) mouse tissues was hybridized with a ^{32}P-labeled 6Ckine probe and subjected to autoradiography. BALB/c mice showed 6Ckine expression in various lymphoid and nonlymphoid tissues, whereas *plt* mice showed expression only in nonlymphoid tissue. Total spleen RNA (20 μg) from BALB/c mice was included as a positive control in each blot. Ethidium bromide–stained gel of BALB/c and *plt* RNA is shown in B and D, respectively.

are present and, in fact, both forms have been reported and characterized in the literature but not recognized as arising from independent genes. Hedrick and Zlotnik (4) reported the mouse 6Ckine-leu form, and Tanabe et al. (3) reported the mouse 6Ckine-ser form; although a direct comparison of the two mouse 6Ckine proteins had not been made, these two reports showed similar findings regarding their chemotactic activity. Furthermore, computer modeling of the differences between the 6Ckine-ser and 6Ckine-leu proteins based on known chemokine crystal structures does not predict any radical differences in the structures of the two proteins (Murgolo, N., and E. Coates, personal communication). An analysis of the human 6Ckine gene shows that it encodes a leucine at position 65 (1, 2, 6), and we were unable to find any ESTs corresponding to a human serine form in any of over 300 ESTs examined (Hedrick, J., and L. Wang, unpublished observation). We cannot, however, formally exclude the possibility of a second human gene.

Chemokine gene duplication is relatively common, and indeed most members of this cytokine superfamily are thought to have arisen through a series of duplications of "primordial" chemokines. The duplication of mouse 6Ckine is likely to have been a relatively recent evolutionary event, as there have been few changes within the exon/intron regions of the genes. It is also possible, however, that some selective pressure has maintained the two genes in a relatively unchanged state, and thus it will be important to determine the relative contributions of each form of 6Ckine to the process of lymphocyte and dendritic cell trafficking in vivo.

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