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Yoichi Kato · Luisa Salter-Cid · Martin F. Flajnik
Chisato Namikawa · Makoto Sasaki · Masaru Nonaka

Duplication of the MHC-linked *Xenopus* complement factor *B* gene

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Abstract We have previously reported the molecular cloning of the mammalian major histocompatibility complex (MHC) class III gene, complement *factor B* (*Bf*) from *Xenopus laevis*, and linkage of the gene to the frog MHC. Here, we estimated the copy number of the *Xenopus Bf* gene by genomic Southern blotting analysis and demonstrated that *Xenopus laevis* has two copies of the *Bf* gene. Both genes co-segregated with the MHC-linked *HSP70* genes among 19 offspring of an *f/r* × *f/r* cross, indicating a close linkage of the two *Bf* genes to the frog MHC. Both genes are transcribed and contain open reading frames. When compared with the previously determined cDNA sequence (*Xenopus Bf A*), the predicted amino acid sequence of the second cDNA species (*Xenopus Bf B*) shows 82% overall identity. Polymerase chain reaction analysis indicated that all of the partially inbred frogs with the *f, r, g*, and *j* MHC haplotypes, as well as 12 outbred frogs tested have both *Bf* genes, suggesting that the duplicated *Bf* genes are stable genetic traits in *Xenopus laevis*.

Introduction

Complement factor B (Bf) plays a pivotal role in the activation of the alternative complement pathway as a catalytic subunit of the alternative pathway C3 convertase, C3bBb (Reid and Porter 1981). Bf and its classical pathway homologue, C2, show the same domain structure, composed of three SCR (short consensus repeat) domains, a von Willebrand domain, and a serine protease domain (Bentley et al. 1986; Ishikawa et al. 1990; Horiuchi et al. 1993). Moreover, *Bf* and *C2* genes have the same exon-intron organization (Ishikawa et al. 1990), suggesting that these two genes were generated by duplication from a common ancestor. There are other well-known examples of gene duplication in the mammalian complement system such as *C3/C4/C5* (Sottrup-Jensen et al. 1985; Wetsel et al. 1988) or *C1r/C1s* (Journet and Tosi 1986; Leytus et al. 1986), indicating that gene duplication was important in establishing the modern complement system. Although it has not been clearly shown when the duplication events occurred during vertebrate evolution, cyclostomes, the most primitive extant vertebrates, seem to have only the alternative complement pathway (Nonaka et al. 1984), most probably representing the situation before the *C3/C4* (Nonaka et al. 1992) or *Bf/C2* (Nonaka et al. 1994) gene duplication. In contrast, cartilaginous fish and higher vertebrates have both the alternative and classical pathways (Dodds and Day 1993), suggesting that these gene duplications occurred between the divergence of cyclostomes and the divergence of cartilaginous fish from the main line of the vertebrate evolution.

The mammalian *Bf* and *C2* genes and the two *C4* genes reside in the major histocompatibility complex (MHC) class III region (Carroll et al. 1984). In addition to these complement genes, the mammalian MHC class III region contains many other diverse genes apparently not structurally or functionally related to the class I and class II genes. Some of them, such as inducible members of the heat shock protein-70 (*HSP70*) family of genes (Milner and Campbell 1990) and cytokine *TNFA* and *TNFB* genes (Carroll et al. 1987), encode proteins involved in immunity. Other genes whose products have no apparent immune function, such as the microsomal enzyme *21-hydroxylase* (Carroll et al. 1985) and *valyl-tRNA synthetase* genes (Hsieh and Campbell 1991), are also located in the class III region. Therefore, the mammalian MHC class III region seems to be constituted of genes whose linkage to class I and class II may have some physiological or evolutionary significance, and of genes present there simply by "accident". Phylogenetic studies of the MHC class III region of the lower

The nucleotide sequence data reported in this paper have been submitted to the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases and have been assigned the accession number D49373

Y. Kato · C. Namikawa · M. Sasaki · M. Nonaka (✉)
Department of Biochemistry, Nagoya City University Medical School, Mizuho-Ku, Nagoya 467, Japan

L. Salter-Cid · M. F. Flajnik
Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL 33101, USA

vertebrates are necessary to elucidate the original genes in this region.

We have previously reported the isolation of *Bf* cDNA clones from *Xenopus laevis* (*X. laevis*) (Kato et al. 1994). The comparison of the deduced amino acid sequence of *Xenopus* Bf with mammalian Bf, C2, and lamprey Bf sequences indicated that the *Bf/C2* gene duplication most likely occurred before the mammalian/amphibian divergence. Moreover, the *Xenopus Bf* gene analyzed was closely linked to the MHC class Ia and *HSP70* loci, indicating that the *Xenopus Bf* gene resides within or in close proximity to the frog MHC (Kato et al. 1994).

X. laevis is a pseudotetraploid (Kobel and Du Pasquier 1986) and all MHC genes examined to date including class I (Shum et al. 1993), class II (Sato et al. 1993), and *HSP70* (Salter-Cid et al. 1994) appear to have been "diploidized". Thus, we set out to determine whether the *Bf* genes are also linked only to the functional MHC and are not found on other chromosomes in which vestigial MHC genes were silenced or deleted.

Materials and methods

Restriction enzymes were purchased from Toyobo (Osaka, Japan) and New England Biolabs (Beverly, MA). The ligation kit was from Takara (Kyoto, Japan). [α-^{32}P]dCTP was from Amersham Japan (Tokyo, Japan). The primer cycle sequencing kit for automated DNA sequencer was from Aplied Biosystems Japan (Tokyo, Japan) and the *Taq* cycle sequencing kit was from Takara. The TA-cloning kit was from Invitrogen (San Diego, CA). Outbred *X. laevis* were purchased from Nippon Bio-Supplies Center, (Nagoya, Japan). Partially inbred MHC-typed *Xenopus* frogs with the haplotypes *f*, *g*, and *r* were bred and maintained at the University of Miami School of Medicine (Miami, FL). Partially inbred frogs with the *j* MHC haplotype were provided by S. Tochinai (Hokkaido University, Japan).

Southern hybridization

Genomic DNA isolation was performed as previously described (Salter-Cid et al. 1994). To prepare Southern blots, 1 to 2 μg of genomic DNA was digested to completion for 3 h with 10 to 30 units/μg DNA of restriction endonucleases. Digested DNA was electrophoresed in a 1% agarose gel and transferred to a nylon membrane. After UV cross-linking, membranes were prehybridized for 10 min at 65 °C in 3 × standard sodium chloride (SSC) (1 × SSC is 150 mM, 15 mM sodium citrate)], 20 min at 65 °C in 3 × SSC, 10 × Denhardt's solution (1 × Denhardt's solution is 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), and 30 min at 65 °C in 10 × Denhardt's solution, 1 M sodium chloride, 50 mM Tris, 10 mM ethylenediaminetetraacetate, 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg/ml denatured salmon sperm DNA. Membranes were then hybridized with radiolabeled probes prepared using Megaprime DNA labeling systems (Amersham) at 65 °C for 16 to 20 h. After hybridization, membranes were washed twice for 30 min in 0.1 × SSC, 0.1% SDS at 65 °C.

Nucleotide sequence analysis

DNA sequencing was performed by the dideoxy chain termination method (Sanger et al. 1977) using a Shimadzu DSQ-1000 or an Applied Biosystems 373A DNA sequencer. Each sequence was determined from both strands.

PCR amplification of genomic DNA segment corresponding to the 5′ untranslated (UT) region

PCR primers were synthesized to amplify the 5′UT region where the *Xenopus* Bf A and *Xenopus* Bf B cDNA sequences show a 24 base pair (bp) size difference. The sense strand primer was TATCATCCTTATCTGCTC and the antisense primer was TTTCTCCCCA(C/A)TCTTTTA. The PCR template was genomic DNA from *X. laevis*. Thirty cycles of amplification were carried out in an Astec PC700 thermocycler (Fukuoka, Japan) using the following parameters: 95 °C for 0.5 min, 50 °C for 1 min, and 72 °C for 1 min.

Results

Copy number of the Xenopus Bf gene

The copy number of the *Xenopus Bf* gene was estimated by Southern hybridization with genomic DNA from frogs with the MHC haplotypes *r/r* and *f/f*. Southern hybridization was performed using an approximately 1.2 kilobase (kb) long 3′-end fragment of the *Xenopus Bf* cDNA (*Xenopus Bf A*) as a probe as described previously [(probe 3′) (Kato et al. 1994)]. Probe 3′ detected two major hybridizing bands with both *r/r* and *f/f* DNA digested with *Hin* dIII (Fig. 1B). The rationale to determine whether these two bands represent two adjacent fragments of a single gene or fragments from two different genes is outlined in Figure 1A. Probe 3′ is divided into two fragments, 3′-a and 3′-b, and are used as hybridization probes separately. If the original two bands are from a single gene, only one fragment (in this case 3′-a) can detect the original two bands and the other fragment (in this case 3′-b) will hybridize to only one band (Fig. 1A, case 1). On the other hand, if the original two bands are from two different genes (Fig. 1A, case 2), both fragments should detect these two bands. As shown in Figure 1B, the same two hybridizing bands detected by probe 3′ were also detected by both probes 3′-a and 3′-b, demonstrating that these two bands represent different genes. Since partially inbred frogs were used for this analysis, these two genes most likely represent a *Bf* gene duplication rather than two different alleles at the single locus. A similar analysis was performed using an approximately 0.6 kb long 5′ end fragment of the *Xenopus Bf* cDNA [(*Xenopus Bf A*) (Fig. 1C)]. The result showed that the 5′ end of the *Xenopus Bf* gene is also duplicated (Fig. 1C). Therefore, the entire *Bf* gene seems to be duplicated in *X. laevis*.

Two Xenopus Bf genes are linked to the frog MHC

We have previously shown that a *Xenopus Bf* gene is linked to the frog MHC (Kato et al. 1994). To test the possible linkage of the other *Bf* gene to the *Xenopus* MHC, we first searched for a combination of restriction enzymes and probes that would detect polymorphisms in the two genes present in the *f* and *r* MHC haplotypes. The combination of *Hin* dIII and probe 3′, used in Figure 1B, fulfilled the above criteria and was used in the mapping study. Although the lower band of the *r* haplotype and the upper band of the *f*

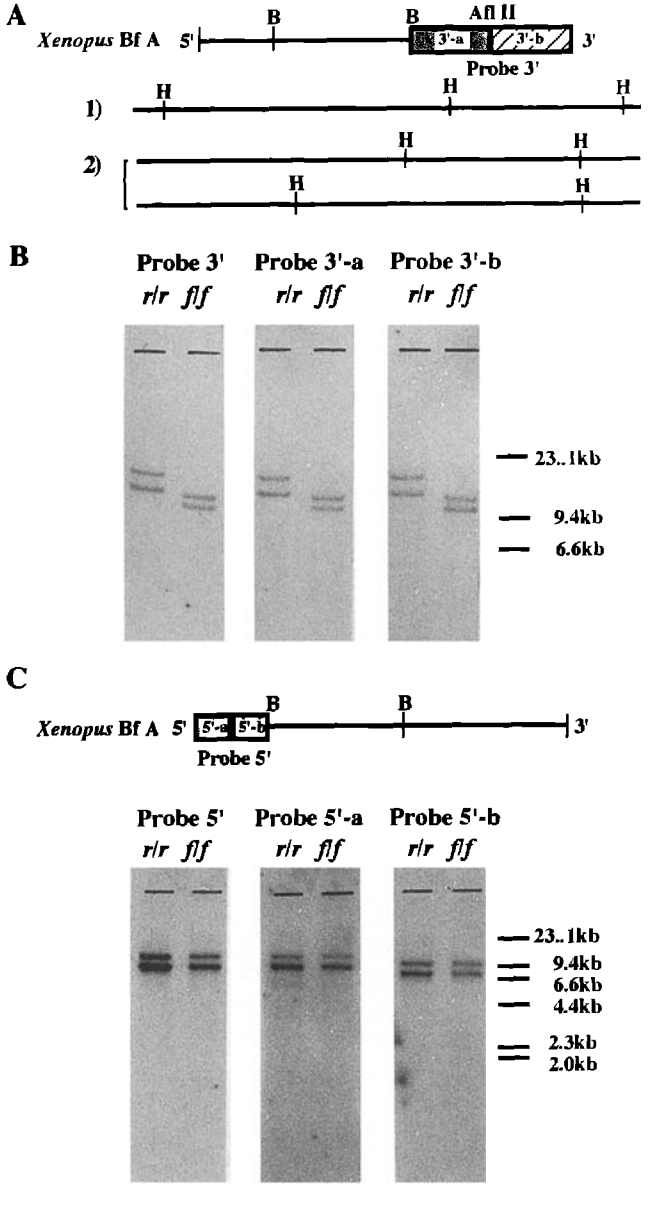


Fig. 1 A–C Genomic Southern blot analysis to estimate copy number of the *Xenopus Bf* gene. **A** Schematic explanation of the rationale for copy number estimation. The two possibilities that the double bands detected by probe 3′ represent 1) two adjacent fragments of a single gene, or 2) two fragments of two different genes are schematically shown. Subfragments of probe 3′, probes 3′-a and 3′-b, were prepared by *Afl* II digestion of probe 3′. **B** Southern hybridization using probes 3′, 3′-a, and 3′-b. One or two micrograms of genomic DNA from frogs with the MHC haplotypes *r/r* and *f/f* were digested with *Hin* dIII. The MHC haplotypes are shown above each lane. Probe 3′, an approximately 1.2 kb long 3′ end fragment of the *Xenopus Bf A* cDNA clone, detected double bands from *r/r* and *f/f* DNA. Probe 3′-a and probe 3′-b detected the same double bands as the bands detected by probe 3′, indicating that possibility #2 of **A** is the actual case. **C** Copy number analysis with the 5′ probe. The same analysis was performed using the 5′ end 0.6 kb fragment of *Xenopus Bf A* as a probe (Probe 5′). The 5′ and 3′ half of probe 5′ were PCR-amplified using primers TAATACGACTCACTATA (T7 promoter) and TTTCGGGTATGGGTATTT (+148 ~ +165 of *Xenopus Bf A*), AAATACCCATACCCGAAA (+148 ~ +165 of *Xenopus Bf A*) and ATTTAGGTGACACTATA (SP6 promoter), respectively. The *r/r* and *f/f* DNA were digested by *Eco* RI

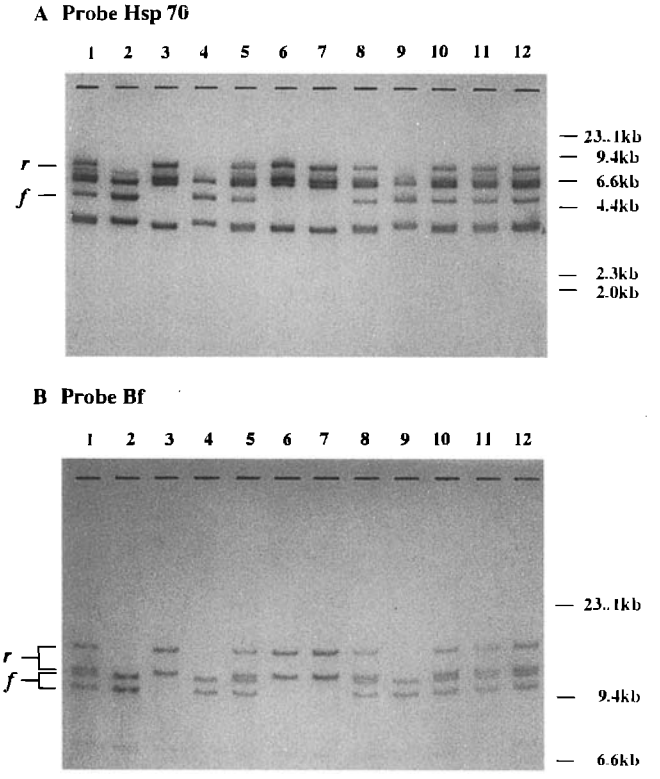
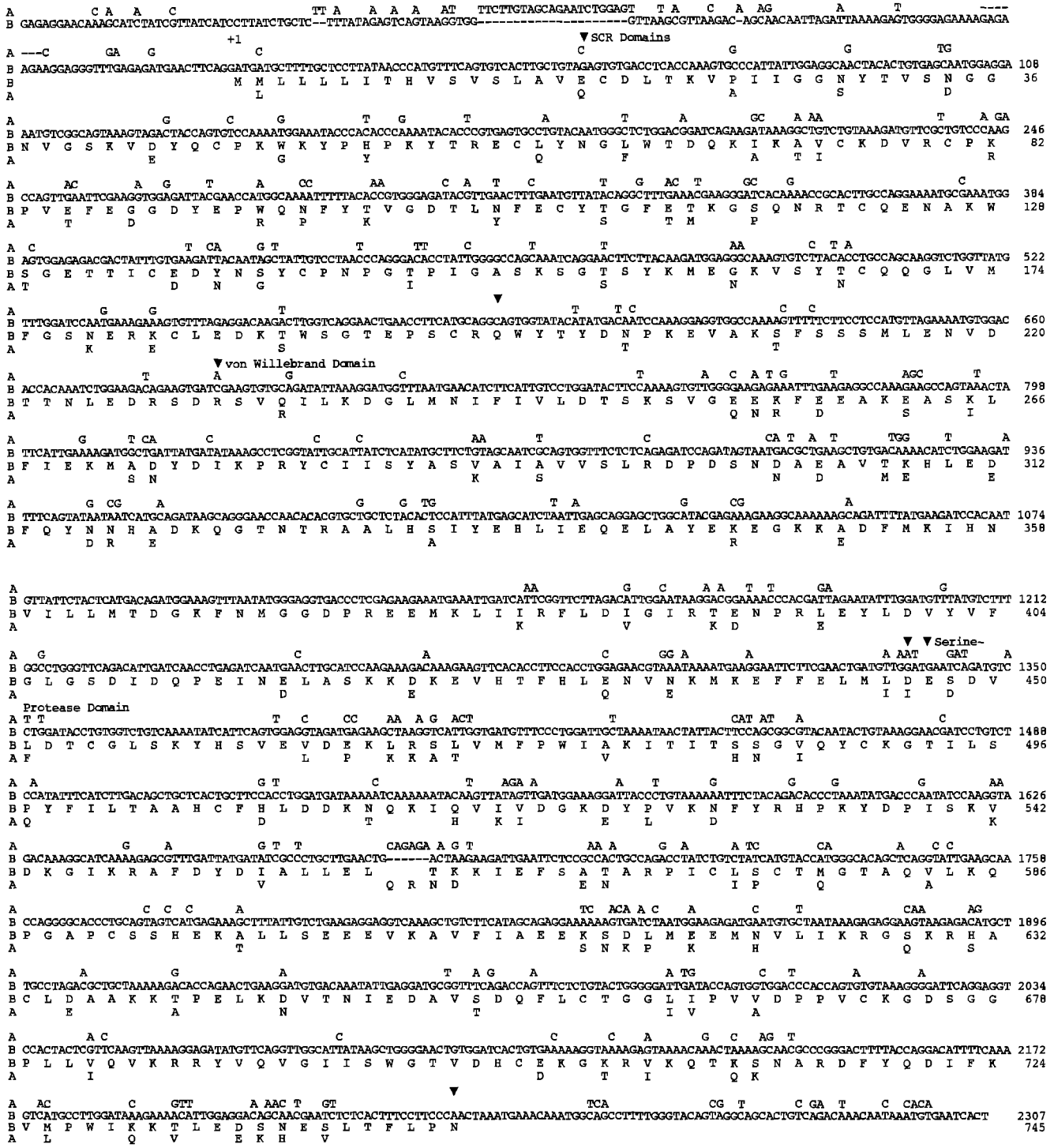


Fig. 2 A, B Southern blot analysis of genomic DNA from members of the *f/r* × *f/r X. laevis* family. **A** MHC typing of the *f/r* × *f/r* offspring was performed using the *HSP70* probe as described (Salter-Cid et al. 1994). Genomic DNA were digested with *Pst* I. The *r* and *f* diagnostic bands described in the previous paper (Salter-Cid et al. 1994) are indicated by *bars* on the *left side*. Haplotypes determined based on the *HSP70* types are: *f/f*, 2, 4, 9; *r/r*, 3, 6, 7; *f/r*, 1, 5, 8, 10, 11, 12. **B** *Bf* typing of the same individuals as used in **A**. Genomic DNA were digested with *Hin* dIII and hybridized with the *Xenopus Bf* probe, probe 3′, as described in the legend to Figure 1. The *r* and *f* diagnostic bands are indicated by *bars* on the *left side*

haplotype showed a similar mobility, there is a slight difference in the size of these two bands (see Figure 2). The MHC haplotypes of 19 offspring from an *f/r* × *f/r* cross were determined by Southern blot hybridization using as a probe the *HSP70* gene, which has been shown to be closely linked to the *Xenopus* MHC (Salter-Cid et al. 1994). The same offspring were then typed for the two *Bf* genes (Fig. 2). Twelve representative offspring shown in Figure 2 were typed as *f/f* (2, 4 ,9), *r/r* (3, 6, 7), and *f/r* (1, 5, 8, 10, 11, 12) with the *HSP70* probe (Fig. 2A). With the *Bf* probe, probe 3′, 20 kb and 13 kb *Hin* dIII bands were *r*-specific, and the 12 kb and 10 kb *Hin* dIII bands were specific for the *f* haplotype (Fig. 2B), indicating that the two *Bf* genes and the *HSP70* genes are closely linked. In total 3 *f/f*, 7 *r/r*, 9 *f/r* individuals were identified with the *HSP70* probe, and the *Bf Hin* dIII polymorphic bands co-segregated in all cases with the *HSP70* typing. These results indicate that the two *Xenopus Bf* genes are linked to each other and reside near or within the *Xenopus* MHC.



Isolation of another Xenopus Bf cDNA clone

In our previous paper (Kato et al. 1994), only one *Bf* cDNA species (*Xenopus Bf A*) was isolated. To examine for expression of the second gene as mRNA, the *Xenopus* liver cDNA library used in the previous study was screened with probe 5′. One point two million plaques of once-amplified library were screened and about sixty positive signals were detected. Twenty-two clones were plaque purified, and restriction enzyme mapping analysis of

Fig. 3 Nucleotide and deduced amino acid sequence of *Xenopus* Bf B. The entire nucleotide sequence of the clone 10 insert is shown together with the deduced amino acid sequence. The nucleotide and deduced amino acid sequences of *Xenopus* Bf A (Kato et al. 1994) are shown above and below the *Xenopus* Bf B sequences, respectively, only at the positions where these two sequences diverge. The initiation methionine codon was assigned from alignment with other Bf and C2 sequences (Fig. 4), and is marked as +1. The nucleotide and amino acid numbers of the *rightmost* residues, starting from the initiation codon, are shown for each lane. The boundaries of the SCR, von Willebrand, and serine protease domains, in this order from the N-terminus, are indicated by *closed triangles*. These domains are assigned based on alignment with the *Xenopus* Bf A sequences. The 32 amino acids between the SCR and von Willebrand domains correspond to an unidentified domain

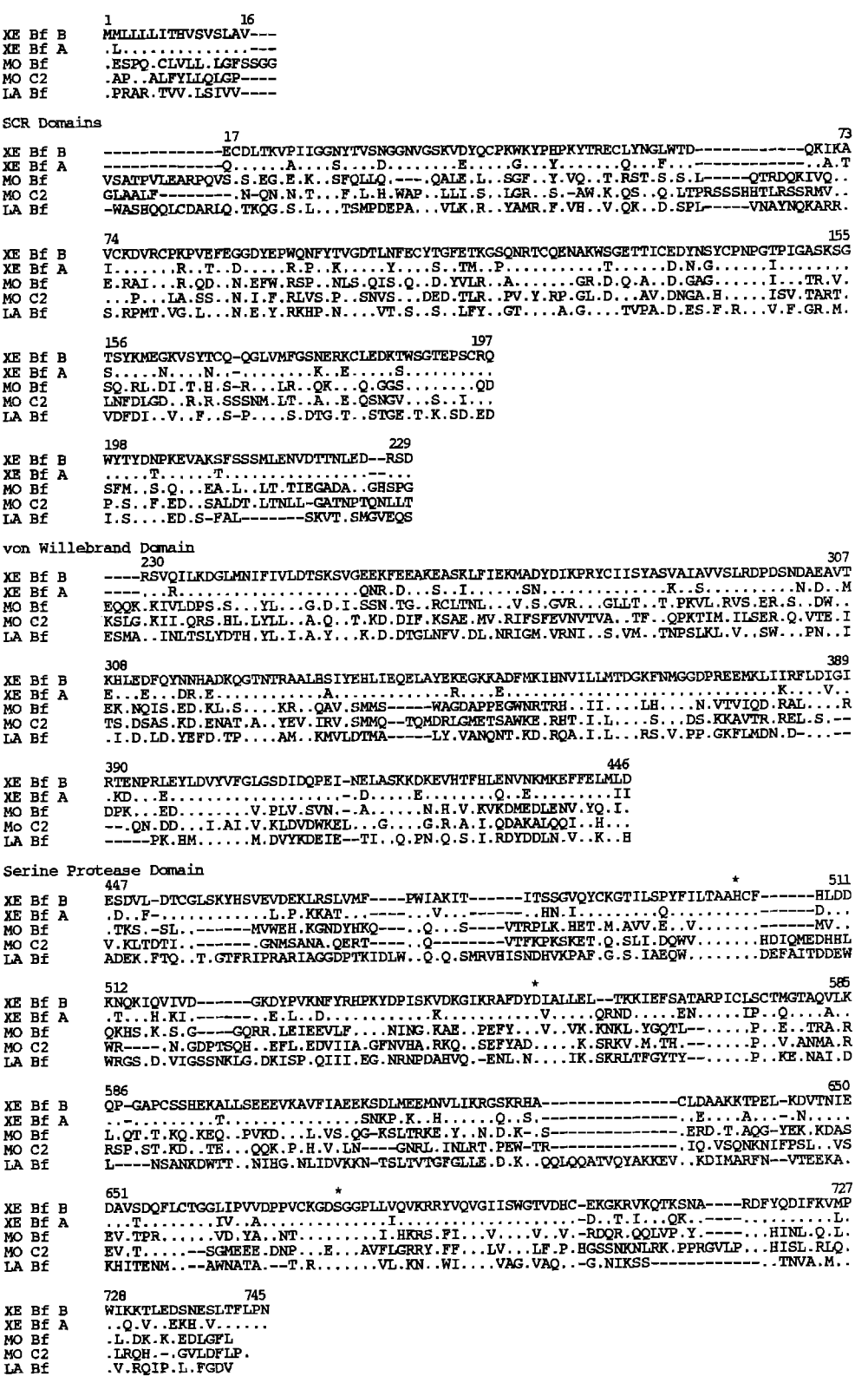


Fig. 4 Alignment of the *Xenopus* Bf B amino acid sequence with the *Xenopus* Bf A, mouse Bf, mouse C2, and lamprey Bf sequences. For each domain, the *Xenopus* Bf B amino acid sequence (XE Bf B) was manually aligned with the *Xenopus* Bf A (XE Bf A), mouse Bf (MO Bf), mouse C2 (MO C2), and lamprey Bf (LA Bf) sequences, based primarily on the positions of the cysteine residues. Identical residues with the *Xenopus* Bf B in the *Xenopus* Bf A, mouse Bf, mouse C2, and lamprey Bf sequences are shown by *dots*. Gaps introduced to increase the identity are shown by *dashes*. Alignment of the signal peptide (amino acid residues 1–16) is presented before the SCR domain. Alignment of the unidentified domain (amino acid residues 198–229) is presented at the end of the SCR domain. The residues at the active center of serine proteases are shown by *asterisks*

these clones indicated the presence of two different cDNA species. Twelve clones had the same restriction map as the previously reported *Xenopus Bf A*. The other ten clones had a different restriction map, and these cDNA species were named *Xenopus Bf B*. Clone 10, containing the longest insert among the latter group, was analyzed for its complete nucleotide sequence and was compared with *Xenopus Bf A* (Fig. 3). The *Xenopus* Bf A and *Xenopus* Bf B sequences are 86% and 82% identical at the nucleotide and amino acid levels, respectively. The nucleotide substitutions between

Table I A calculated regional amino acid identity among the *Xenopus* Bf B, *Xenopus* Bf A, mouse Bf, mouse C2, and lamprey Bf sequences. Percent amino acid identity among the *Xenopus* Bf B (XE Bf B), *Xenopus* Bf A (XE Bf A), mouse Bf (MO Bf), mouse C2 (MO C2), and lamprey Bf (LA Bf) sequences was calculated based on the alignment shown in Figure 4. Number of identical residues per number of compared positions is shown in parentheses

	SCR domains	von Willebrand domain	Serine protease domain	Total
XE Bf B vs XE Bf A	81% (147/181)	85% (184/217)	79% (239/301)	82% (615/747)
XE Bf B vs MO Bf	43% (86/201)	39% (86/221)	38% (118/309)	39% (305/784)
XE Bf B vs MO C2	40% (79/200)	31% (68/222)	29% (93/324)	31% (250/796)
XE Bf B vs LA Bf	40% (80/200)	33% (73/221)	24% (82/339)	30% (245/810)

the *Xenopus* Bf A and *Xenopus* Bf B sequences are distributed almost uniformly over the entire length, indicating that these two cDNA species are not generated by differential processing of transcripts from a single gene but are products of two distinct genes. Since only two genes have been detected by the Southern hybridization analysis with genomic DNA from all frogs under stringent hybridization and/or washing conditions, we conclude that these two cDNA species correspond to these two *Bf* genes.

Alignment of the Xenopus Bf B amino acid sequence with Xenopus Bf A, mouse Bf, mouse C2, and lamprey Bf sequences

The predicted *Xenopus* Bf B amino acid sequence was manually aligned with the *Xenopus* Bf A, mouse Bf, mouse C2 (Ishikawa et al. 1990), and lamprey Bf (Nonaka et al. 1994) sequences (Fig. 4). The domain structure, including three SCR domains, a von Willebrand domain, and a serine protease domain from the N-terminus, is completely conserved among these proteins. The calculated regional amino acid identity based on this alignment among *Xenopus* Bf B, *Xenopus* Bf A, mouse Bf, mouse C2, and lamprey Bf is shown in Table I. In total, *Xenopus* Bf B has 82%, 39%, 31%, and 30% amino acid identity with *Xenopus* Bf A, mouse Bf, mouse C2, and lamprey Bf, respectively.

Duplicated Bf genes are widely recognized among partially inbred and outbred Xenopus laevis

To examine whether the duplicated *Bf* genes are found only in the few partially inbred strains and outbred frogs tested or are rather widely distributed in *X. laevis*, PCR amplification of the region where *Xenopus Bf A* and *Xenopus Bf B* show a size difference was performed using genomic DNA from partially inbred frogs of the *f, r, g,* and *j* haplotypes as well as twelve outbred frogs. PCR primers were designed in the 5′UT region, which would amplify a 110 bp and an

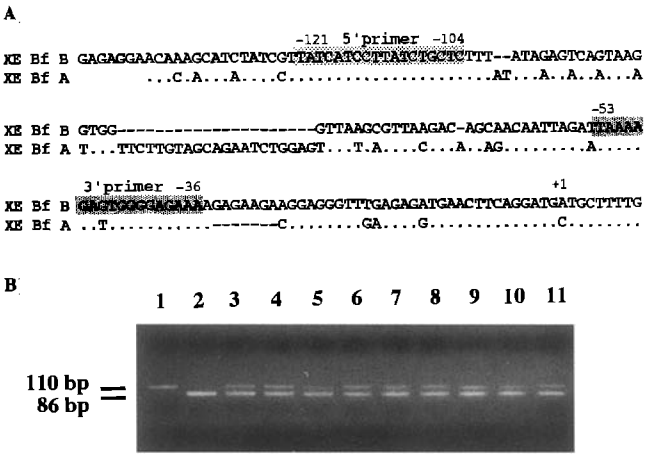


Fig. 5A, B RT-PCR amplification of the different length fragment of the 5′UT region of the *Xenopus Bf B* and *Xenopus Bf A*. **A** 5′UT region sequences of *Xenopus Bf B* and *Xenopus Bf A* are shown. The *Xenopus Bf A* residues identical with the *Xenopus Bf B* are shown by *dots*. Deletion introduced into the *Xenopus Bf B* and *Xenopus Bf A* sequences for better alignment are shown by *dashes*. Sequences of the primers used for PCR amplification are indicated by *differential shading*. **B** PCR amplification of genomic DNA of partially inbred and outbred frogs; lanes 1 and 2, control amplification of cDNA clones, clone 9 (*Xenopus Bf A*) and clone 10 (*Xenopus Bf B*), respectively: lane 3, *r/r*: lane 4, *f/f*: lane 5, *g/g*: lane 6, *j/j*: lanes 7 ~ 11, outbred frogs. The two bands of the expected sizes, 86 bp and 110 bp, are indicated

86 bp band from the *Xenopus Bf A* and *Xenopus Bf B* cDNAs, respectively (Fig. 5A). The two expected bands were amplified from the inbred and outbred frogs (Figure 5B and data not shown), indicating that all frogs have both *Bf* genes and that no intron is inserted between the two sequences corresponding to these two primers. Nucleotide sequence analysis of these bands confirmed that the 86 bp band corresponded to the 5′UT region of *Xenopus Bf B* and the 110 bp bands corresponded to the 5′UT region of *Xenopus Bf A* (data not shown). This result demonstrated that the duplicated *Bf* genes are a widely distributed, stable genetic trait in *X. laevis*.

Discussion

Here we demonstrated that *X. laevis* has the duplicated *Bf* genes with an 82% deduced amino acid sequence identity, and that both these duplicated genes are closely linked to the frog MHC. Although information at the protein level is still missing, both genes produce transcripts with open reading frames and thus are likely to be functional. Since the *Bf* and *C2* genes are believed to be generated from a common ancestor by gene duplication, we examined the possibility that one of these two *Xenopus Bf* genes is a frog counterpart of the mammalian *C2* gene. This possibility is highly improbable, however, because the similarity between *Xenopus* Bf A and *Xenopus* Bf B (82%) is too high when compared with the similarity between mouse Bf and C2 [(38%) (Ishikawa et al. 1990)]. Moreover, both *Xenopus* Bf A and *Xenopus* Bf B amino acid sequences showed a

higher similarity to the mammalian Bf sequences than to the mammalian C2 sequences (39%–40% identity to Bf; 30%–31% identity to C2). Thus, our data do not support the idea that one of the *Xenopus Bf* genes corresponds to the mammalian *C2* gene.

Although *Bf* gene duplication in any species has not been reported, other examples of complement gene duplication in the MHC class III region are well known. Two human *C4* (Carroll et al. 1984), named *C4A* and *C4B*, show more than 99% amino acid sequence identity (Belt et al. 1984), and both proteins possess C4 functional activity, although there is a slight difference in chemical reactivity between them (Dodds et al. 1986). In the mouse (Chaplin et al. 1983), two *C4* genes, *C4* and sex-limited protein *Slp*, are 95% identical in amino acid sequences (Nonaka et al. 1986), and Slp is reported to be non-functional due to a three amino acid deletion in the region critical for proteolytic activation by Cls (Ogata et al. 1989). Although Slp is expressed only in some inbred and wild mice (Klein 1975; Shreffler et al. 1984), its structural gene seems to be stable and no deletion of the *C4* or *Slp* gene has been reported. In contrast, duplicated human *C4* genes seem to be rather unstable, and deletion and further duplication are fairly common (Collier et al. 1989). Duplicated *X. laevis Bf* genes were detected from all frogs analyzed, indicating that these duplicated genes are stable. Although the physiological significance (if any) of duplication of the *Bf* genes is unclear, it is possible that the Bf A and Bf B proteins, with only 82% amino acid identity, have different reactivities with C3.

X. laevis is a pseudotetraploid species, and the globin, actin, and vitellogenin genes are encoded by two active genetic loci (Jeffreys et al. 1980; Stutz and Spohr 1986; Schubiger and Wahli 1986). The *Xenopus* MHC loci, in contrast, are believed to be functionally diploidized (Kobel and Du Pasquier 1986). The class I, class II, and *HSP70* genes appear to have been diploidized by deletion (Salter-Cid et al. 1994). Thus, it was possible that one of the *Bf* genes may have been encoded an another chromosome, perhaps in a vestigial MHC. Linkage analysis, however, demonstrated that both *Bf* genes are closely linked to each other and to the frog MHC, suggesting that duplication of the *Bf* gene occurred in the functional MHC. The data presented here on the *Bf* genes, as well as our unpublished observation that the *Xenopus LMP7* gene is a single copy gene closely linked to the frog MHC (Namikawa and coworkers unpublished data), support the idea that the *Xenopus* MHC has been diploidized by the deletion of the entire complex.

Hughes and Hughes (1993) estimated, based on the described method (Nei and Gojobori 1986), the numbers of synonymous nucleotide substituions per synonymous site (d_S) and nonsynonymous substituions per nonsynonymous site (d_N) of 17 *X. laevis* genes considered to be products of tetraploidization, and found the average d_S/100 sites and d_N/100 sites to be 19.2 ± 1.7 and 2.5 ± 0.5, respectively. The corresponding values estimated for the two *Bf* genes were 23.7 ± 2.5 and 10.1 ± 0.8, respectively. Since the d_S value of the two *Bf* genes is similar to that of the average, *Bf* gene duplication seems to have occurred around the time of tetraploidization. It is possible that the whole genome was unstable just after tetraploidization, and a non-reciprocal recombination may have generated duplicated *Bf* genes in the MHC. Physical analysis of the two *Bf* genes and a study of *Bf* genes in other *X. species*, now in progress in our laboratory, will help to clarify the molecular details and hence the underlying mechanism of *Bf* gene duplication.

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References

Belt, K. T., Carroll, M. C., and Porter, R. R. The structural basis of the multiple forms of human complement component C4. *Cell 36:* 907–914, 1984

Bentley, D. R. Primary structure of human complement component C2. *Biochem J 239:* 339–345, 1986

Carroll, M. C., Campbell, R. D., Bentley, D. R., and Porter, R. R. A molecular map of the human major histocompatibility complex class III region linking complement genes C4, C2 and factor B. *Nature 307:* 237–241, 1984

Carroll, M. C., Campbell, R. D., and Porter, R. R. Mapping of steroid 21-hydroxylase genes adjacent to complement component C4 genes in HLA, the major histocompatibility complex in man. *Proc Natl Acad Sci USA 82:* 521–525, 1985

Carroll, M. C., Katzman, P., Alicot, E. M., Koller, B. H., Geraghty, D. E., Orr, H. T., Strominger, J. L., and Spies, T. Linkage map of the human major histocompatibility complex including the tumor necrosis factor genes. *Proc Natl Acad Sci USA 84:* 8535–8539, 1987

Chaplin, D. D., Woods, D. E., Whitehead, A. S., Goldberger, G., Colten, H. R., and Seidman, J. G. Molecular map of the murine S region. *Proc Natl Acad Sci USA 80:* 6947–6951, 1983

Collier, S., Sinnott, P. J., Dyer, P. A., Price, D. A., Harris, R., and Strachan, T. Pulsed field gel electrophoresis identifies a high degree of variability in the number of tandem 21-hydroxylase and complement C4 gene repeats in 21-hydroxylase deficiency haplotypes. *EMBO J 8:* 1393–1402, 1989

Dodds, A. W. and Day, A. J. The phylogeny and evolution of the complement system. *In* K. Whaley, M. Loos, J. M. Weiler (eds.): *Complement in Health and Disease,* pp. 39–88, Kluwer Academic Publishers, London, 1993

Dodds, A. W., Law, S.-K. A., and Porter, R. R. The purification and properties of some less common allotypes of the fourth component of human complement. *Immunogenetics 24:* 279–285, 1986

Horiuchi, T., Kim, S., Matsumoto, M., Watanabe, I., Fujita, S., and Volanakis, J. E. Human complement factor B: cDNA cloning, nucleotide sequencing, phenotypic conversion by site-directed mutagenesis and expression. *Mol Immunol 30:* 1587–1592, 1993

Hsieh, S.-J. and Campbell, R. D. Evidence that gene G7a in the human major histocompatibility complex encodes valyl-tRNA synthetase. *Biochem J 278:* 809–816, 1991

Hughes, M. K. and Hughes, A. L. Evolution of duplicate genes in a tetraploid animal, *Xenopus laevis. Mol Biol Evol 10:* 1360–1369, 1993

Ishikawa, N., Nonaka, M., Wetsel, R. A., and Colten, H. R. Murine complement C2 and factor B genomic and cDNA cloning reveals different mechanisms for multiple transcripts of C2 and B. *J Biol Chem 265:* 19040–19046, 1990

Jeffreys, A. J., Wilson, V., Wood, D., Simons, J. P., Kay, R. M., and Williams, J. G. Linkage of adult α- and β-globin genes in *X. laevis* and gene duplication by tetraploidization. *Cell 21:* 555–564, 1980

Journet, A. and Tosi, M. Cloning and sequencing of full-length cDNA encoding the precursor of human complement component C1r. *Biochem J 240:* 783–787, 1986

Kato, Y., Salter-Cid, L., Flajnik, M. F., Kasahara, M., Namikawa, C., Sasaki, M., and Nonaka, M. Isolation of the *Xenopus* complement factor B complementary DNA and linkage of the gene to the frog MHC. *J Immunol 153:* 4546–4554, 1994

Klein, J. A case of no sex-limitation of Slp in the murine H-2 complex. *Immunogenetics 2:* 297–299, 1975

Kobel, H. R. and Du Pasquier, L. Genetics of polyploid *Xenopus*. *Trends Genet 2:* 310–315, 1986

Leytus, S. P., Kurachi, K., Sakariassen, K. S., and Davie, E. W. Nucleotide sequence of the cDNA coding for human complement C1r. *Biochemistry 25:* 4855–4863, 1986

Milner, C. M. and Campbell, R. D. Structure and expression of the three MHC-linked *HSP70* genes. *Immunogenetics 32:* 242–251, 1990

Nei, M. and Gojobori, T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol Biol Evol 3:* 418–426, 1986

Nonaka, M., Fujii, T., Kaidoh, T., Natsuume-Sakai, S., Nonaka, M., Yamaguchi, N., and Takahashi, M. Purification of a lamprey complement protein homologous to the third component of the mammalian complement system. *J Immunol 133:* 3242–3249, 1984

Nonaka, M., Nakayama, K., Yu, D. Y., and Takahashi, M. Complete nucleotide and derived amino acid sequences of sex-limited protein (Slp), nonfunctional isotype of the fourth component of mouse complement (C4). *J Immunol 136:* 2989–2993, 1986

Nonaka, M. and Takahashi, M. Complete complementary DNA sequence of the third component of complement of lamprey. *J Immunol 148:* 3290–3295, 1992

Nonaka, M., Takahashi, M., and Sasaki, M. Molecular cloning of a lamprey homologue of the mammalian MHC class III gene, complement factor B. *J Immunol 152:* 2263–2269, 1994

Ogata, R. T., Cooper, N. R., Bradt, B. M., Mathias, P., and Picchi, M. A. Murine complement component C4 and sex-limited protein: identification of amino acid residues essential for C4 function. *Proc Natl Acad Sci USA 86:* 5575–5579, 1989

Reid, K. B. M. and Porter, R. R. The proteolytic activation systems of complement. *Annu Rev Biochem 50:* 433–464, 1981

Salter-Cid, L., Kasahara, M., and Flajnik, M. *Hsp70* genes are linked to the *Xenopus* major histocompatibility complex. *Immunogenetics 39:* 1–7, 1994

Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA 74:* 5463–5467, 1977

Sato, K., Flajnik, M. F., Du Pasquier, L., Katagiri, M., and Kasahara, M. Evolution of the MHC: isolation of class II β-chain cDNA clones from the amphibian *Xenopus laevis*. *J Immunol 150:* 2831–2843, 1993

Schubiger, J.-L. and Wahli, W. Linkage arrangement in the vitellogenin gene family of *Xenopus laevis* as revealed by gene segregation analysis. *Nucleic Acid Res 14:* 8723–8734, 1986

Shreffler, D. C., Atkinson, J. P., Chan, A. C., Karp, D. R., Killion, C. C., Ogata, R. T., and Rosa, P. A. The C4 and Slp genes of the complement region of the murine H-2 major histocompatibility complex. *Phil Trans R Soc Lond B 306:* 395–403, 1984

Shum, B. P., Avila, D., Du Pasquier, L., Kasahara, M., and Flajnik, M. F. Isolation of a classical MHC class I cDNA from an amphibian. *J Immunol 151:* 5376–5386, 1993

Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Lønbland, P. B., Jones, C. M., Wierzbicki, D. M., Magnusson, S., Domdey, H., Wetsel, R. A., Lundwall, Å., Tack, B. F., and Fey, G. H. Common evolutionary origin of α2-macroglobulin and complement components C3 and C4. *Proc Natl Acad Sci USA 82:* 9–13, 1985

Stutz, F. and Spohr, G. Isolation and characterization of sarcomeric actin genes expressed in *Xenopus laevis* embryos. *J Mol Biol 187:* 349–361, 1986

Wetsel, R. A., Lemons, R. S., Le Beau, M. M., Barnum, S. R., Noack, D., and Tack, B. F. Molecular analysis of human complement component C5: localization of the structural gene to chromosome 9. *Biochemistry 27:* 1474–1482, 1988