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The genes for the inter-α-inhibitor family share a homologous organization in human and mouse

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Abstract. Inter-α-inhibitor (*IαI*) and related molecules in human are comprised of three evolutionarily related, heavy (H) chains and one light (L) chain, also termed bikunin. The latter originates from a precursor molecule that is cleaved to yield the bikunin and another protein designated α-1-microglobulin (A1m). The four H and L chains are encoded by four distinct genes designated *H1, H2, H3,* and *L*. The *L* and *H2* genes are localized onto human chromosomes (chr) 9 and 10, respectively, whereas the *H1* and *H3* genes are tandemly arranged on chr 3.

Mouse poly(A)$^+$ RNAs or endonuclease-restricted mouse DNA were analyzed by standard and pulsed-field gel electrophoresis (PFGE) techniques in agarose gels and blot-hybridized with human *H1, H2, H3* or *L* cDNA probes. The variable sized transcripts and unique restriction fragment patterns detected with each probe indicate that four genes, including one common *L* gene for A1m and bikunin also exist in mouse. The co-migration of *H1*- and *H3*-hybridizing fragments on PFGE suggests that the mouse *H1* and *H3* genes are also tandemly arranged. An *Msp* I restriction fragment length polymorphism (RFLP) in the mouse *L* gene (proposed symbol, *Intin-4*) links this gene to other genes already mapped at mouse Chr 4 near the brown (*b*) locus, a homologous region to the human chr 9q32-34 band where the human *IαI L* gene is located. Therefore, a similar number and arrangement of *IαI* genes is found in mouse and human, including the triplication of an *H* gene ancestor. These results point to an ancient origin of this complex set of genes.

Introduction

IαI is a plasma glycoprotein of high Mr that acts as a serine protease inhibitor (reviewed in Gebhard and Hochstrasser 1986; Salier 1990). Characteristic amino acid sequences in the protease inhibitory domains of *IαI* indicate that this protein belongs to the superfamily of Kunitz-type protease inhibitors (Wachter and Hochstrasser 1979). However, the function of *IαI* in vivo still awaits complete elucidation and this function may not, or not only, be related to its protease inhibitory capacity (McKeehan et al. 1986; Salier 1990). Moreover, the recent characterization of a family of *IαI*-related molecules in plasma suggests that more than one function could pertain to *IαI* family members (Salier 1990). The complex set of mRNAs and genes that encode the *IαI* family has recently been described in human (Bourguignon et al. 1985; Kaumeyer et al. 1986; Salier et al. 1987; Gebhard et al. 1988; Vetr et al. 1989; Diarra-Mehrpour et al. 1989, 1990); the information helped clarify the multipolypeptide chain structure of *IαI* and related molecules (Bourguignon et al. 1983; Jessen et al. 1988; Enghild et al. 1989). One L chain and three H chains are separately synthesized in the liver by four mRNAs which are designated *L, H1, H2* and *H3,* respectively (Salier et al. 1987; Diarra-Mehrpour et al. 1989; Enghild et al. 1989). The approximate sizes of the L or H chain mRNAs are 1.25 or 3.2 kb, respectively. In human, the corresponding *L* and *H2* genes have been mapped by in situ hybridization to 9q32-33 and 10p14-15, respectively, whereas both *H1* and *H3* genes map to 3p21.1-21.2 (Diarra-Mehrpour et al. 1989). The three *H* genes are evolutionarily related and they most likely originate from the *H3* gene as an ancestor (Diarra-Mehrpour et al. 1989). Separate exons in the gene for the L chain precursor code for two tandem, in-frame proteins, termed α-1-microglobulin (A1m) and bikunin that are released by post-translational cleavage of their common L chain pre-

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cursor (Kaumeyer et al. 1986; Diarra-Mehrpour et al. 1990). The mature A1m molecule, another plasma glycoprotein, is neither structurally nor functionally related to the *IαI* family. In contrast, the bikunin contains two Kunitz-type protease inhibitory domains (Wachter and Hochstrasser 1979), hence its name (Gebhard et al. 1990a), and is found in all *IαI* family members studied to date (Enghild et al. 1989). *IαI* itself is made of the *H1, H2* and bikunin chains, whereas other family members in plasma include: (1) pre-α-inhibitor (*PαI*) made of the H3 chain and bikunin; and (2) the so-called *IαI* derivatives which are free bikunin molecules (Enghild et al. 1989; reviewed in Salier 1990).

IαI and *IαI* derivatives have been found in the plasma of all mammalian species studied so far, from rodents to human (reviewed in Gebhard and Hochstrasser 1986), whereas the recently discovered *PαI* molecule has not yet been investigated in various species. However, at the present time, *IαI* cDNAs and genes have been studied almost exclusively in human. Given the complexity in organization and evolution of human *IαI* genes, we were interested in studying whether or not such complexity is a recent evolutionary event. Our present results indicate that the overall organization of the four *L* and *H* genes existed prior to the divergent evolution of human and mouse.

Materials and methods

Human cDNA probes

The cDNA clones for L, H1, H2 or H3 chains of human *IαI* were obtained from a human liver cDNA library cloned in the λ gt11 expression vector (Salier et al. 1987). Most of these clones have been described elsewhere (Salier et al. 1987; Diarra-Mehrpour et al. 1989). Briefly, an *Ava* I-*Ava* I, 591 bp A1m-encoding cDNA and an *Ava* I-*Ava* I, 501 bp bikunin-encoding cDNA from clone λ HuLITI-23 (Salier et al. 1987) were used together (*L* probe) or separately, depending on the experiments (see Results). Together, these probes cover 87% of the full-length L chain cDNA. The *H1* cDNA probe was the *Eco*R I-*Eco*R I, 2186 bp insert of clone λ HuHITI-11000 (M. Diarra-Mehrpour and J.-P. Salier, unpublished results) covering about 70% of the full-length *H1* cDNA from its 3′ end. The *H2* cDNA probe was the *Eco*R I-*Eco*R I, 1140 bp insert of clone λ HuHITI-9 (Salier et al. 1987) covering about the central 35% of the full-length *H2* cDNA. The *H3* cDNA probe was a mixture of an *Eco*R I-*Bam*H I, 1485 bp segment and a *Taq* I-*Taq* I, 772 bp segment that are contiguous within the insert from clone λ HuHITI-131 (Diarra-Mehrpour et al. 1989); together both segments cover about 70% of the full-length *H3* cDNA from its 3′ end.

Mouse strains

Mice of both sexes were at least 35 days old when used. A/J (A), C57BL/6J (B), AXB F_1 and BXA F_1 were purchased from The Jackson Laboratory and maintained in the University of Michigan animal quarters. DNA from recombinant inbred (RI) lines between the A/J and C57BL/6J strains (AXB, BXA) (Nesbitt and Skamene 1984) were provided by M. Nesbitt, San Diego, Calif., while the computerized strain distribution pattern (SDP) for these RI lines is maintained by B. Paigen, Bar Harbor, Me. DNA from other inbred strains was purchased from The Jackson Laboratory.

Mouse mRNAs

Poly(A)$^+$ mRNAs were purified from mouse liver by an acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi 1987). They were electrophoresed along with an RNA ladder (0.24–9.5 kb from Gibco-BRL) in a denaturing formaldehyde/agarose gel following standard procedures. They were then blotted overnight in 15× SSPE (20× SSPE is 3 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4) onto an Hybond N nylon membrane (Amersham) and cross linked by UV exposure according to Reed and Mann (1985).

Genomic DNA isolation and restriction

For standard electrophoresis in agarose gel, genomic DNA was isolated from mouse spleens or livers (fasted for 24 h) by the usual phenol/chloroform extractions and ethanol precipitation and stored in a Tris 10 mM, EDTA 1 mM, pH 7.5 buffer. For PFGE, genomic DNA from freshly isolated mouse spleens was prepared and stored in agarose blocks to prevent any shearing as already described (Verga and Erickson 1990).

The purified DNAs kept in solution or in agarose blocks were digested overnight to completion with at least ten units restriction endonuclease (from Boehringer-Mannheim or New England Biolabs) per μg DNA. DNA blocks for PFGE were equilibrated in restriction enzyme buffer prior to digestion, and re-equilibrated in Tris 10 mM, EDTA 1 mM pH 8.0 prior to electrophoresis.

DNA electrophoreses and blotting

Mouse total genomic DNA was electrophoresed in a horizontal 0.8% agarose gel in a 1× TBE (10× TBE is 900 mM Tris, 900 mM borate, 10 mM EDTA buffer pH 8.2) at 2–3 V/cm for 16 h. The 1 kb DNA ladder from Gibco-BRL was used as a size marker. Alternatively, PFGE for human or mouse genomic DNA was performed in a Pulsaphor (Pharmacia-LKB) with an hexagonal electrode. The following conditions were used: 1% agarose gel in 0.25× TBE, 150 V, 90 mA, 65 s switch time, for 41 h at 12°C. Concatamers of phage λ (Pharmacia) were used as size markers.

After electrophoresis, the agarose gel was stained with ethidium bromide and photographed. The gel was soaked (2 × 10 min) in HCl 0.15 N for DNA nicking and then it was soaked in 0.5 N NaOH for 30 min, and blotted in the same NaOH solution for 18–24 h onto a GeneScreen (Dupont) or a Bio-Trace RP (Gelman) nylon membrane. The membrane was then neutralized in 2× SSPE for 5 min and air dried.

Hybridizations

All cDNA probes were labeled with (α^{32}P) dCTP (3000 Ci/mmole, Amersham) to a specific activity of 0.5–2.10^9 cpm/μg by the random oligonucleotide procedure.

Hybridizations of RNA or DNA filters were carried out at 42°C for 16–48 h in 30% deionized formamide, 50 mM Tris pH 7.5, 1 M NaCl, 10% dextran sulfate, 0.1% SDS, 10× Denhardt's solution (50× solution is 1% polyvinylpyrrolidone, 1% ficoll, 1% bovine serum albumin), 100 μg/ml heat-denatured herring sperm DNA. Post-hybridization washes were done in 2× SSPE, 0.5% SDS at room temperature (4 × 5 min) and at 60°C (2 × 1 h). The membrane was then autoradiographed onto an X-ray film (XAR-5, Kodak) with intensifying screen for 1–10 days at −80°C. Prior to re-probing, the RNA membranes were de-hybridized by a 5 min wash in a boiling solution of 1% SDS and a second wash in the same solution at 65°C for 30 min, and stored dry. The DNA membranes were de-hybridized at room temperature by washes in NaOH 0.4 N (2 × 15 min) and in 2× SSPE, 0.5% SDS (2 × 15 min), then re-exposed for checking the de-hybridization efficiency, and dried for storage.

Results

Analysis of mouse liver poly(A)$^+$ RNAs

Human *H1, H2, H3,* A1m or bikunin cDNA probes were hybridized to nylon membranes that contained

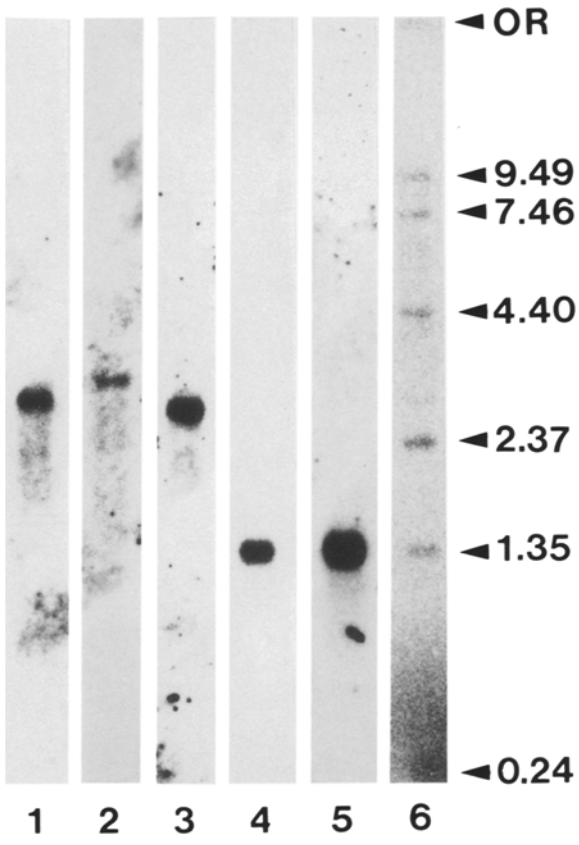

Fig. 1. RNA blot analysis of poly(A)$^+$ RNAs from mouse liver. Aliquots of 1 μg poly(A)$^+$ RNAs were separated in a denaturing agarose gel, blotted, and the resulting nylon strips were separately hybridized with either of various human *IαI* cDNA probes. **Lane 1:** *H1* probe; **lane 2:** *H2* probe; **lane 3:** *H3* probe; **lane 4:** *A1m* (L chain) probe; **lane 5:** bikunin (L chain) probe; **lane 6:** RNA ladder in kb. OR means origin. Anode at the bottom.

electrophoretically separated liver poly(A)$^+$ RNAs from the Swiss mouse strain (the original, non-congenic strain from Lausanne, Switzerland). As shown in Fig. 1 each human probe recognized a population of mouse RNAs: the *H1*, *H2* or *H3* chain probes detected a 3.0, 3.2, or 2.9 kb (±0.1 kb) RNA band, respectively, whereas the L chain probes, namely A1m or bikunin cDNAs, detected the same 1.3 kb band. Using successive hybridizations of a single RNA lane with each of the three H chain probes without intermediate dehybridizations, we precisely verified that the three H chain RNAs indeed differ in sizes (not shown).

Patterns of restriction fragments in mouse IαI genes

Genomic DNA from mouse strains A/J or C57BL/6J was digested with a series of common restriction endonucleases (four or six base cutters) and the pattern of resulting DNA fragments was analyzed by blot hybridization with each of four human *IαI* cDNA probes, namely *H1*, *H2*, *H3* or the complete *L* probe (A1m + bikunin cDNAs). Typical results obtained with several enzymes are summarized in Table 1. Each enzyme/probe combination revealed a characteristic set of restriction fragments and most fragments were found with only one enzyme/probe combination. Overall, the results suggested that the four different cDNA probes detected four different genes.

PFGE fragments of mouse IαI genes

A similar analysis was performed with the genomic DNA from A/J or C57BL/6J mouse strains and restriction enzymes that recognize rare sites in mammalian DNA. The restricted DNAs were separated by PFGE and blotted. The resulting filter was successively hybridized with each of the *IαI* probes. The results are presented in Fig. 2. The *H1* and *H3* probes clearly revealed identical restriction fragments (smallest size: 130 kb with *Ksp* I or *Sal* I) in mouse genomic DNA. Likewise, each of the *L* probes, namely the A1m or bikunin cDNAs, detected another, common set of restriction fragments. Finally, the *H2* probe revealed a third, distinct pattern of fragments. With some of the restriction enzymes tested, each probe did not show a sizeable hybridization band (Fig. 2). In such instances, most likely the *IαI*-associated restriction fragments were quite large and retained within the zone of limited mobility (for example, lanes 3 and 4 in panel b) or were too small for the size range covered by the gel (lane 5 in panel b).

An Msp I polymorphism maps the mouse IαI L gene to Chr 4 near the brown locus

During the analysis of *IαI*-related restriction fragments in mouse DNA, as described above, possible RFLPs

Table 1. Sizes of restriction fragments revealed by various, human *IαI* cDNA probes in genomic DNA from A/J or C57BL/6J mouse strains.[a] Sizes are in kb.

Probe[b]	*H1*	*H2*	*H3*	*L*[c]
Restriction Endonuclease				
Hinc II	1.8/1.2/.9	6.0/3.9/1.9 1.5/1.2/1.1/1.0	6.0/3.2/2.1 1.5/.8	6.0/2.2/1.9
Pst I	4.5/1.8/1.2	6.3/1.3	4.5/2.9/2.5/1.3	2.9/2.4/.9
Pvu II	1.7/1.5/1.35	5.8/4.3/3.8/2.0	5.5/2.5/1.9	4.0/1.7/1.1
Sac I	5.5/2.8/2.5/1.5	4.6/3.7	4.0/3.5/2.8 2.5/1.8	6.0/5.0
Stu I	3.8/1.8	8.0/5.0	3.6/1.8/1.3	4.0/1.85/1.6

[a] The restriction fragments obtained with the five enzymes indicated were identical in both strains.
[b] Described in Materials and methods.
[c] L probe = A1m cDNA + bikunin cDNA.

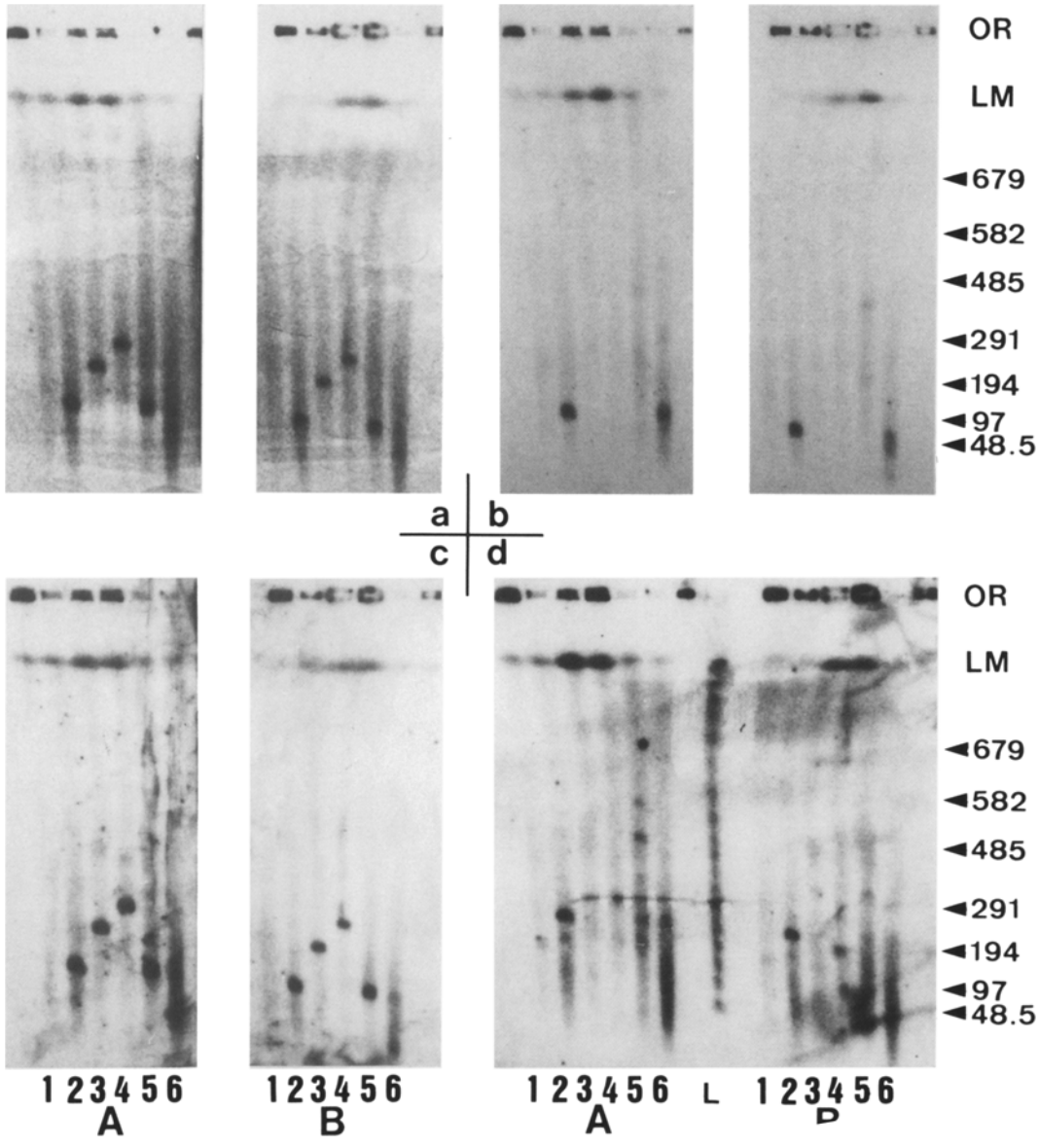

Fig. 2. Large restriction fragments revealed by various, human *Iα/* cDNA probes in mouse genomic DNA separated by PFGE. Genomic DNA from A/J **(panel A)** or C57BL/6J **(panel B)** mouse strains was digested with various endonucleases: *Cla* I **(lane 1)**; *Ksp* I **(lane 2)**; *Mlu* I **(lane 3)**; *Not* I **(lane 4)**; *Sal* I **(lane 5)**; *Sma* I **(lane 6).** The DNA digests were separated in a single PFGE gel and blotted. The resulting membrane was successively hybridized with the following human cDNA probes: *H1* **(panel a)**, *H2* **(panel b)**, *H3* **(panel c)**, *L* (A1m or bikunin cDNAs; **panel d**). Identical results were obtained when using either A1m or bikunin cDNAs and therefore only one corresponding autoradiography is shown. The size markers are shown in **lane L** and the sizes in kb are on the right. OR is origin. LM is zone of limited mobility. Anode at the bottom. Please note: (1) a slight difference in migration velocity between the left and right side of the gel (panel A versus B); and (2) possible *Not* I and *Sal* I RFLPs for the *L* chain gene that are currently the subject of investigations.

were also investigated by comparison of the A/J (A) and C57BL/6J (B) mouse strains. DNAs restricted with a series of 17 restriction enzymes (*Apa* I, *Dra* I, *Eco*R I, *Hae* III, *Hinc* II, *Hind* III, *Hinf* I, *Kpn* I, *Msp* I, *Nci* I, *Pst* I, *Pvu* II, *Rsa* I, *Sac* I, *Sca* I, *Stu* I, *Taq* I) were successively blot-hybridized with each of the three human probes for the *H1, H2, H3* genes and the complete human *L* probe (A1m + bikunin cDNAs). An RFLP for the *L* gene was found with *Msp* I that revealed a 2.6 or 3.0 kb allelic fragment as illustrated in Fig. 3. Further, separate hybridizations with the A1m or bikunin cDNA probes of the L chain gene indicated that this RFLP was detected by the A1m cDNA (Fig. 3). The 2.6 kb genotype was found in the A/J, BALB/cJ and C3H/HeJ mouse strains whereas the 3.0 kb genotype was found in the 129/J, C57BL/6J, C57BL/10J, CBA/J, C58/J, DBA/2J and NZW strains (not shown).

The distribution of this RFLP was determined in all available AXB and BXA RI strains (Fig. 3 and Table 2) and the SDP compared to other polymorphic genes already typed in this RI strain set. Linkage was found to *b*, a coat color gene on Chr 4. There were five mismatches with *b* among 41 strains giving a map distance of 3.7 ± 1.9 cM (95% confidence interval 1.1–10.8 cM) calculated according to Silver and Buckler

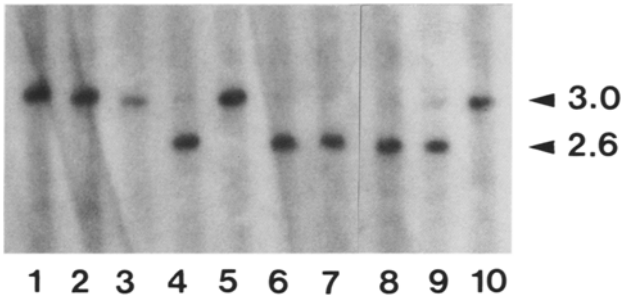

Fig. 3. *Msp* I RFLP in the *IαI* L chain gene of various mouse strains. *Msp* I-restricted DNA from various mouse strains was hybridized with the A1m cDNA probe. This probe detects a 2.6 or 3.0 kb allelic band. The patterns from AXB 1, 3, 4, 5, 12, 17, 25, or BXA 1, 7, 8 RI mouse strains are shown in **lanes 1–10**, respectively. Sizes in kb on the right. Anode at the bottom.

(1986). The *IαI L* gene (proposed symbol *Intin-4*) showed linkage with *Mup-1* (six mismatches in 40 strains for a map distance of 4.9 ± 2.4 cM), but not with *Pmv-23* (16 mismatches in 39 strains), suggesting a gene order of *Mup-1, Intin-4, b* and *Pmv-23*.

Discussion

A very limited number of reports have dealt with *IαI* or other Kunitz-type protease inhibitors in rodents (Kastern et al. 1986; Kido et al. 1988; Fukuchi et al. 1989) and nothing is known about *IαI* genes in non-primate species. Therefore, and given the complexity of *IαI* genes in human, we have been interested in looking at *IαI* gene organization in a rather distant mammalian group to seek insights on the origin and evolution of this set of genes.

In hybridization experiments, five human cDNA probes were used, namely the *H1, H2* and *H3* cDNAs and the two L chain-related A1m- or bikunin-encoding cDNAs. The three H chain cDNAs are evolutionarily related (Diarra-Mehrpour et al. 1989) and the two L chain cDNAs code for distinct proteins that originate from a common L precursor in human (Kaumeyer et al. 1986). Since each of these five human probes could detect a limited number of bands on Southern hybridizations with mouse genomic DNA (Table 1 and Fig. 2), we conclude that nucleotide sequence similarities in *IαI*-related, single-copy genes exist between mouse and human. Despite the evolutionary relationships between the *H1, H2* and *H3* genes, the quite different patterns of bands seen in Northern or Southern blots with the *H1, H2* or *H3* probes indicate that no spurious cross-hybridization between each mouse *IαI* H chain RNA or gene and several of our human probes took place under the hybridization stringency used. We propose mouse gene symbols for *H1, H2* and *H3* of *Intin-1, Intin-2,* and *Intin-3,* with *Intin-4* for the L chain gene.

The same-sized, 1.3 kb mRNA population was detected with both A1m and bikunin probes in mouse liver (Fig. 1), in agreement with previous results obtained in human (Salier et al. 1987) and pig (Gebhard et al. 1990b). Furthermore, a same restriction pattern of mouse DNA was seen when either the A1m or bikunin probe was used to detect restriction fragments in PFGE experiments (Fig. 2). Therefore, we conclude that in mouse, as in human (Kaumeyer et al. 1986; Salier et al. 1987; Vetr et al. 1989; Diarra-Mehrpour et al. 1990), a single gene (or identically duplicated genes) and mRNA code for a L chain precursor molecule comprised of A1m and bikunin. Likewise, A1m in rat is encoded by a 1.25 kb mRNA (Kastern et al. 1986). Given our present results in mouse, it is likely that this rat RNA in fact codes for a tandem A1m/bikunin precursor.

Table 2. Distribution pattern of mouse *IαI L* gene *Msp* I RFLP in AXB and BXA RI strains.

AXB[a]																								
Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	19	20	21	22	23	24	25
Mup-1[b]	B[c]	A	B	B	A	B	B	A	U[d]	B	A	B	A	B	B	A	U	B	B	B	A	B	B	A
																							X	
Intin-4	B	A	B	B	A	B	B	A	B	B	A	B	A	B	B	A	B	B	B	B	A	B	A	A
						X								X						X			X	
b[b]	B	A	B	B	A	A	B	A	B	B	A	B	A	A	B	A	B	B	B	A	A	B	B	A
	X				X		X			X	X						X				X	X		
Pmv-23[b]	A	A	B	B	B	A	A	A	B	A	B	B	A	A	U	U	A	U	B	A	B	A	B	A
BXA[e]																								
Strain	1	2		4			7	8	9		11	12	13	14	16	17	18	19	20		22	23	24	25
Mup-1[b]	A	A		A			A	B	A		U	A	A	B	A	B	A	A	A		A	B	A	B
				X																	X	X	X	X
Intin-4	A	A		B			A	B	A		B	A	A	B	A	B	A	A	A		B	A	B	A
				X																				
b[b]	A	A		A			A	B	A		B	A	A	B	A	B	U	U	A		B	A	B	A
				X			X				X										X	X	X	
Pmv-23[b]	A	A		B			B	B	A		A	A	A	B	U	B	A	A	A		B	B	A	A

[a] RI lines starting with the A/J female by C57BL/6J male F_1.
[b] This locus maps to mouse Chr 4.
[c] A = A/J allele; B = C57BL/6J allele; X = crossover.
[d] Unknown.
[e] RI lines starting with the C57BL/6J female by A/J male F_1.

In the present study we describe for the first time a polymorphism of mouse *IαI L* gene, namely an *Msp* I RFLP. Through linkage analysis in RI lines, this RFLP allowed us to precisely map this *L* gene (*Intin-4*) in very close proximity to the *b* locus on mouse Chr 4. The q34 band of chr 9 in human is homologous to this region of mouse Chr 4 (Searle et al. 1989). Therefore, our results suggest that the human *IαI* L gene is located at 9q34, although it was previously localized at 9q32-q33 by the lower resolution of in situ hybridization onto metaphasic chromosomes (Diarra-Mehrpour et al. 1989).

The human *H1*, *H2* and *H3* probes detected three poly(A)$^+$ RNA populations in mouse liver. The size range (2.9–3.2 kb) measured for these RNA populations is close to the range (3.0–3.3 kb) seen for human *H1*, *H2* and *H3* chain poly(A)$^+$ RNAs (Salier et al. 1987; Diarra-Mehrpour et al. 1989). The human *H1*, *H2* and *H3* probes also revealed distinct sets of restriction fragments in Southern analysis of mouse DNA (Table 1). This suggests that these three probes detected three distinct mouse *H* genes. Furthermore, the *H1* and *H3* probes exhibited the same pattern of bands (130 kb or more) in PFGE experiments, which demonstrates a close, tandem arrangement of the *H1* and *H3* genes within 130 kb in the mouse genome. This is reminiscent of co-localization of the human *H1* and *H3* genes at band 3p21.1-21.2 (Diarra-Mehrpour et al. 1989) where further, recent results indicate that these two genes are tandemly arranged within less than 100 kb (J.-P. Salier, unpublished results).

Overall, our present results show that in mouse as well as in human, *IαI* and/or related molecules are encoded by four discrete genes, including one *L* gene of homologous chromosomal localization, an *H2* gene, and two *H1* and *H3* genes in a tandem arrangement. These four genes are transcribed in the liver. A comparison of amino acid or nucleotide sequence between *H1*, *H2* and *H3* chains or cDNAs in human, has shown that *H3* and *H1* sequences are highly similar and *H1* and *H2* sequences are most distantly related (Diarra-Mehrpour et al. 1989). Therefore, the following order of appearance of *H* chain genes was suggested: (1) the *H2* gene originated from an *H3* ancestor gene; and (2) more recently, *H1* originated from a duplication of the *H3* ancestor gene (Diarra-Mehrpour et al. 1989). Our present observations of homologous organizations of *IαI* genes in human and mouse indicate that such a triplication of the *H3* ancestor gene occurred prior to the divergence of human and mouse from a common ancestor about 80 million years ago. It will be of interest to analyze whether similar amino acid sequences and enzyme specificities of the protease inhibitory sites in the bikunin moiety have been conserved in mouse and human or whether they have extensively diverged despite highly conserved, surrounding sequences. The second event has been reported for other protease inhibitors with a common ancestor, namely human α-1-antichymotrypsin and mouse contrapsin (Hill et al. 1984).

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