Expression of the $\alpha_1$-Proteinase Inhibitor Gene Family During Evolution of the Genus Mus

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$\alpha_1$-Proteinase inhibitors ($\alpha_1$-PIs) are members of the serpin superfamily of proteinase inhibitors, and are important in the maintenance of homeostasis in a wide variety of animal taxa. Previous studies have shown that in mice (genus Mus), evolution of $\alpha_1$-PIs is characterized by gene amplification, region-specific concerted evolution, and rapid accumulation of amino acid substitutions. The latter occurs primarily in the reactive center, which is the region of the $\alpha_1$-PI molecule that determines the inhibitor’s specificity for target proteinases. The P1 residue within the reactive center, which is methionine in so-called orthodox $\alpha_1$-PIs and an amino acid other than methionine in unorthodox $\alpha_1$-PIs, is a primary determinant of inhibitor specificity. In the present study, we find that the expression of mRNAs encoding unorthodox $\alpha_1$-PIs is polymorphic within Mus species, i.e., among individuals or inbred strains. This is in striking contrast to mRNAs that encode orthodox $\alpha_1$-PIs, whose concentrations are relatively invariant. The intraspecies variations in mRNA expression represent polymorphisms in the structure of the $\alpha_1$-PI gene family. The results, taken together with previously described aspects of $\alpha_1$-PI evolution, indicate that the dissimilar levels of polymorphism exhibited by orthodox and unorthodox $\alpha_1$-PIs, which likely have distinct physiological functions, may reflect different levels of selective constraint. The significance of this finding to the evolution of gene families is discussed.

Introduction

The serpin (serine proteinase inhibitor) gene superfamily, which is found in a wide variety of eukaryotic taxa, functions in the maintenance of homeostasis by protecting organisms from the potentially lethal effects of unregulated proteolytic activity (Carrell, Pemberton, and Boswell 1987; Huber and Carrell 1989; Potempa, Korzus, and Travis 1994). Serpins share considerable homology over large evolutionary distances (Hunt and Dayhoff 1980; Huber and Carrell 1989). While they are named for their ability to inhibit serine proteinases, several members of the serpin family, such as corticosteroid-binding protein and angiotensinogen, have no known inhibitory function (Huber and Carrell 1989). Recognition of target proteinases by serpins is governed by a small region, termed the reactive center, that forms a protruding loop extending out from the surface of the polypeptide (Lawrence et al. 1995; Malgorzata et al. 1995; Wright 1996). The P1 residue within the reactive center is particularly important in determining target specificity (Gettins, Patston, and Schapira 1993; Schechter et al. 1993; Potempa, Korzus, and Travis 1994; Wright 1996).

One of the more abundant serpins in the mammalian bloodstream is $\alpha_1$-proteinase inhibitor ($\alpha_1$-PI, also known as antitrypsin), whose primary role is protection of the lung from the proteolytic action of neutrophil elastase (Travis and Salvesen 1983; Carrell, Pemberton, and Boswell 1987). Indeed, low circulating $\alpha_1$-PI levels have severe pathological consequences, involving excessive proteolytic degradation of elastin fibers in the lung and, eventually, a severe form of emphysema (Carrell 1986; Brantley, Nukiwa, and Crystal 1988). Based upon the identity of the P1 residue, $\alpha_1$-PIs have been classified into two groups. $\alpha_1$-PIs with methionine at the P1 position are designated as orthodox inhibitors, while those with amino acids other than methionine at the P1 site are designated as unorthodox inhibitors (Suzuki et al. 1991; Goto et al. 1994; Ray, Gao, and Ray 1994). Orthodox $\alpha_1$-PIs are present in all species, and represent the primary inhibitor of elastase. Unorthodox inhibitors are found predominantly in species containing multiple $\alpha_1$-PI genes (Borriello and Krauter 1990, 1991; Suzuki et al. 1991; Goto et al. 1994; Goodwin, Baumann, and Berger 1996). In several cases, it has been shown that the unorthodox $\alpha_1$-PIs have biochemical properties that are different from those of orthodox inhibitors (Suzuki et al. 1991; Goto et al. 1994; Ray, Gao, and Ray 1994). Thus, they are likely to have distinct physiological functions.

The evolutionary history of serpins has been of considerable interest. Several investigators have observed that the amino acid sequence of the reactive center loop is hypervariable among species (Hill and Hastie 1987; Laskowski et al. 1987; Borriello and Krauter 1990, 1991; Inglis and Hill 1991; Ohta 1994; Goodwin, Baumann, and Berger 1996). This hypervariability appears to be a consequence of unusually high rates of nonsynonymous substitution within the reactive center regions (Hill and Hastie 1987; Borriello and Krauter 1990, 1991; Rheumne et al. 1994; Goodwin, Baumann, and Berger 1996). Recent studies have shown that, in addition to having hypervariable reactive centers, mammals vary in the number of $\alpha_1$-PI genes within their genomes. Some species, such as human and bovine, have a single $\alpha_1$-PI gene (Kurachi et al. 1981; Long et al. 1984; Brown et al. 1989; Sinha, Bakhshi, and Kirby 1992), while others, such as mouse, have as many as five genes (Hill et al. 1985; Borriello and Krauter 1991; Goodwin, Baumann, and Berger 1996). Small gene families have also been found in the rabbit and guinea pig (Suzuki et al. 1991; Ray, Gao, and Ray 1994; Saito and
Sinohara 1995). Several investigations have shown that \( \alpha_1 \)-PI gene transcription can differ within and among these families. For example, one of four \( \alpha_1 \)-PIs in the rabbit is induced during an acute phase response in the liver and in blood monocytes (Ray, Gao, and Ray 1994, 1995); this induction most likely is a consequence of an NFkB site in the promoter (Ray, Gao, and Ray 1995). In \textit{Mus caroli}, the single orthodox \( \alpha_1 \)-PI transcript is expressed at high levels in the kidney, representing a change in the tissue-specificity of \( \alpha_1 \)-PI gene transcription (Berger and Baumann 1985; Latimer, Berger, and Baumann 1987, 1990; Rheume et al. 1988).

To date, studies of the \( \alpha_1 \)-PI gene family have focused on comparisons within families and among species. The hypervariable nature of the reactive center raises interesting questions concerning intraspecies \( \alpha_1 \)-PI gene polymorphism. Is the \( \alpha_1 \)-PI family polymorphic within species, or is it fixed in structure and expression? Are all members of the family similarly polymorphic or fixed? What is the role of selection in determining the presence or lack of \( \alpha_1 \)-PI gene family polymorphism? In the present paper, we have begun to address these questions by examining the expression of specific \( \alpha_1 \)-PI genes within species.

### Materials and Methods

#### Northern Blot Analysis

All mice used in this study were obtained from sources that have been described previously (Rheume et al. 1994). Extraction of liver RNA was performed by the guanidinium hydrochloride method (Cox 1968). For Northern blotting, RNAs (10 \( \mu \)g/sample) were fractionated through 1.5% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes, and fixed to the membranes by UV-crosslinking (Stratagene). The membranes were incubated overnight at 42°C in a solution containing 6 \( \times \) SSC (1 \( \times \) SSC = 0.015 M sodium chloride), 0.15 M sodium citrate, pH 7.0; 0.15 M sodium chloride), 1 \( \times \) Denhardt’s solution, 100 \( \mu \)g/ml herring testes DNA, 0.5% sodium dodecyl sulfate, and a \( 32^P \)-end-labeled nucleotide probe. The probes, which are complementary to the reactive center regions of various \( \alpha_1 \)-PI mRNAs (Goodwin, Baumann, and Berger 1996; Rheaume et al. 1994). Among the 60 clones, one hybridized to the conserved region (nucleotides 459-479) of \textit{Mus \alpha_1 \,-PI} mRNAs (Goodwin, Baumann, and Berger 1996); the other membrane was hybridized to a mixture of probes corresponding to the reactive centers of four \textit{M. saxicola}-specific mRNAs (SAX1–4; see Table 1). Amplification of \( \alpha_1 \)-PI Genes

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>DOM1</td>
<td>5'-CATAGGAACCGCTTGGTAGAGA-3'</td>
</tr>
<tr>
<td>DOM2</td>
<td>5'-CATAGGAACCGCTTGGTAGAGA-3'</td>
</tr>
<tr>
<td>DOM3</td>
<td>5'-ATAAGGAGCCTTGGTAGAGA-3'</td>
</tr>
<tr>
<td>DOM4</td>
<td>5'-ATAAGGAGCCTTGGTAGAGA-3'</td>
</tr>
<tr>
<td>DOM5</td>
<td>5'-CAAAAACCGCCTTGGTAGAGA-3'</td>
</tr>
<tr>
<td>DOM6</td>
<td>5'-AACGGCTTAGAGCTGTTAGA-3'</td>
</tr>
<tr>
<td>CAR</td>
<td>5'-CATAGGGAACCGCTTGGTAGAGA-3'</td>
</tr>
<tr>
<td>SAX1</td>
<td>5'-CATAGGGAACCGCTTGGTAGAGA-3'</td>
</tr>
<tr>
<td>SAX2</td>
<td>5'-ATGATACCCCTAAAGCAGT-3'</td>
</tr>
<tr>
<td>SAX3</td>
<td>5'-CTTCTACCCCTTTCAAGCAGA-3'</td>
</tr>
<tr>
<td>SAX4</td>
<td>5'-ATAGAGGAGCCTTGGTAGAGA-3'</td>
</tr>
<tr>
<td>NOTE.—All probes are complementary to the reactive centers of the indicated mRNAs; they do not span identical regions within the reactive centers. DOM1–6 are from \textit{M. domesticus} (Hill et al. 1984; Borriello and Krauter 1991). CAR is from \textit{M. caroli} (Latimer, Berger, and Baumann 1990). SAX1–4 are from \textit{M. saxicola} (Goodwin, Baumann, and Berger 1996).</td>
<td></td>
</tr>
</tbody>
</table>

Isolation of a Novel \( \alpha_1 \)-PI mRNA from \textit{M. saxicola}

To identify cDNAs corresponding to \( \alpha_1 \)-PI mRNAs of \textit{M. saxicola}, a mini-library of cDNA clones was prepared using DNA fragments generated by reverse transcription–polymerase chain reaction (RT-PCR), as described previously (Goodwin, Baumann, and Berger 1996). The upstream primer (5'-GGCAATTCTCTTACGGTAGGTGTTTAGTTTAGTGGT-3') was complementary to nucleotides 424–443 of SAX1 mRNA, while the downstream primer (5'-GCGAATTCTCTTACGGTAGGTGTTTAGTTTAGTGGT-3') was complementary to nucleotides 1235–1255 (Goodwin, Baumann, and Berger 1996); each primer contained an EcoRI site at its distal end to facilitate cloning. Products of RT-PCR were ligated into the EcoRI site of the vector PBS-SK(-) (Stratagene), and 60 independent clones were isolated and affixed onto duplicate nitrocellulose membranes. One of the membranes was hybridized to a 32P-end-labeled oligonucleotide probe (5'-CTGAAGCTGTTAGGTGAGAAGT-3') that corresponds to a conserved region (nucleotides 459–479) of \textit{Mus \alpha_1 \,-PI} mRNAs (Goodwin, Baumann, and Berger 1996); the other membrane was hybridized to a mixture of probes corresponding to the reactive centers of four \textit{M. saxicola}-specific mRNAs (SAX1–4; see Rheume et al. 1994). Among the 60 clones, one hybridized to the conserved probe, but not to the mixture of \textit{M. saxicola} probes. This clone defined a novel \( \alpha_1 \)-PI, termed SAX5 (see text).
DOM4-specific primers, while a product of length 79 bp was expected with the DOM6-specific primer.

**DNA Sequencing**

Sequencing was by the dideoxy chain-termination method, using the Sequenase version 2.0 kit (U.S. Biochemicals, Cleveland, OH). Synonymous substitution rates \( (d_s, \) i.e., the number of synonymous substitutions per synonymous site) and nonsynonymous substitution rates \( (d_N, \) i.e., the number of nonsynonymous substitutions per nonsynonymous site) were calculated by the method of Nei and Gojobori (1986), using the MEGA suite of computer programs (Kumar, Tamura, and Nei 1993).

**Results**

**\( \alpha_1 \)-PI Expression in Inbred Strains of \( M. \) domesticus**

In previous work, cDNAs corresponding to 12 mouse \( \alpha_1 \)-PI mRNAs, including six from \( M. \) domesticus inbred strains C57BL/6J and BALB/c, four from the wild-derived species \( M. \) saxicola, and one each from \( M. \) caroli and \( M. \) minutoides, were isolated and analyzed (Hill et al. 1984; Latimer, Berger, and Baumann 1990; Borriello and Krauter 1990, 1991; Rheaume et al. 1994; Goodwin, Baumann, and Berger 1996). These efforts provided insights into the complex evolutionary history of the \( \alpha_1 \)-PI gene family, which involves gene amplification, domain-specific concerted evolution, and accelerated divergence of the reactive center. The cDNA clone from strain BALB/c, which was the first mouse \( \alpha_1 \)-PI to be isolated, is distantly related to the five \( \alpha_1 \)-PI transcripts of strain C57BL/6J (Goodwin, Baumann, and Berger 1996), suggesting the existence of intraspecies variation in \( \alpha_1 \)-PI mRNA expression. Therefore, the nature and extent of variability in \( \alpha_1 \)-PI gene expression among inbred strains of \( M. \) domesticus were examined in more detail.

The expression patterns for individual \( \alpha_1 \)-PI mRNAs were compared in six strains of \( M. \) domesticus by Northern blot analysis of liver RNA. Specific oligonucleotides that are complementary to the highly divergent reactive center regions and that have been shown to discriminate among the various \( \alpha_1 \)-PI mRNAs of strain C57BL/6J (Borriello and Krauter 1990) were used to quantitate each mRNA. The oligonucleotides were hybridized to blots containing RNA from inbred strains C57BL/6J, A/J, AKR/J, DBA/2J, C3H/HeJ, and 129/J; RNA from \( M. \) caroli served as a negative control, since this species has a single \( \alpha_1 \)-PI mRNA that does not hybridize to any of the available \( M. \) domesticus-specific reactive center probes (unpublished data). Four of the six \( \alpha_1 \)-PI mRNAs exhibited considerable variability in expression among the six inbred strains tested. Two classes of strains could be identified (fig. 1). C57BL/6J-like strains (C57BL/6J, A/J, and C3H/HeJ) are characterized by high expression of the DOM3-, DOM4-, and DOM5-specific mRNAs and little or no expression of the DOM6-specific mRNA. DBA/2J-like strains (DBA/2J, AKR/J, and 129/J) RNA from \( M. \) caroli served as a negative control, since this species has a single \( \alpha_1 \)-PI mRNA that does not hybridize to any of the available \( M. \) domesticus-specific reactive center probes (unpublished data). Four of the six \( \alpha_1 \)-PI mRNAs exhibited considerable variability in expression among the six inbred strains tested. Two classes of strains could be identified (fig. 1). C57BL/6J-like strains (C57BL/6J, A/J, and C3H/HeJ) are characterized by high expression of the DOM3-, DOM4-, and DOM5-specific mRNAs and little or no expression of the DOM6-specific mRNA. DBA/2J-like strains (DBA/2J, AKR/J, and 129/J) exhibit abundant expression of the DOM6-specific mRNA and little if any expression of the DOM3-, DOM4-, and DOM5-specific mRNAs. The DOM1 and DOM2 mRNAs were relatively invariant among the six strains (fig. 1). Of note is the fact that all four mRNAs displaying variable expression among strains encode unorthodox \( \alpha_1 \)-PIs, whereas the two invariant mRNAs encode orthodox \( \alpha_1 \)-PIs.
The interstrain differences in α₁-PI mRNA concentrations were consistently observed with a number of independent RNA samples collected over a 10-year period (data not shown). Thus, variability in levels of unorthodox α₁-PI expression among inbred strains appears to be the result of genetic, rather than environmental or physiological, variation.

Variable Expression of Unorthodox α₁-PI mRNAs in M. saxicola

Since the generation of inbred strains involves fixation of variant alleles from a wild population, we investigated the possibility that expression of the α₁-PI gene family might also vary among individuals of a wild-derived Mus species. In previous work (Rheaume et al. 1994; Goodwin, Baumann, and Berger 1996), we identified four α₁-PI mRNAs (SAX1, SAX2, SAX3, and SAX4) in the species M. saxicola. Three of these (SAX1, SAX2, and SAX3) encode orthodox α₁-PIs, while one (SAX4) encodes an unorthodox inhibitor (Rheaume et al. 1994). We examined the expression of these mRNAs in livers of over 40 individual M. saxicola mice to determine if their concentrations varied in a manner that is similar to what was observed in M. domesticus. The SAX1, SAX2, and SAX3 mRNAs, all of which encode orthodox inhibitors (Rheaume et al. 1994; Goodwin, Baumann, and Berger 1996) showed little variation in expression among individual mice. In contrast, only about half of the mice expressed SAX4, which encodes an unorthodox inhibitor (Rheaume et al. 1994; Goodwin, Baumann, and Berger 1996). These results are summarized in figure 1. Thus, as in M. domesticus, M. saxicola exhibits highly variable expression of unorthodox, but not orthodox, α₁-PI mRNAs.

α₁-PI mRNA Expression in Additional Mus Species

To examine the expression patterns of previously characterized α₁-PI mRNAs in a broad array of Mus species, oligonucleotide probes corresponding to the reactive centers of several Mus species were used to measure mRNA levels in the livers of M. hortulanus, M. caroli, M. cervicolor, M. cookii, M. pahari, and M. minutoides. The results are summarized in figure 1. The dendogram at the far left of the table indicates the consens phylogeny for the Mus species (Bonhomme and Guénet 1989; She et al. 1990; Catzeflis, Aguilar, and Jaegar 1992). In general, each probe was species-specific, i.e., each oligonucleotide hybridized specifically to α₁-PI mRNA of the species from which it was obtained. Additional observations are of interest. It is apparent that more reactive centers exist than have been characterized thus far within the genus Mus, since none of the probes hybridized to mRNA from M. cervicolor or M. pahari (fig. 1). Thus, these two species may express α₁-PI mRNAs with distinct reactive centers. Also, some reactive center motifs are present in more than one species. For example, the M. caroli-specific oligonucleotide hybridized to mRNA from M. cookii, but not to that from the more closely-related M. cervicolor (fig. 1). Several M. domesticus-specific probes (DOM1, DOM2, DOM4, and DOM5) hybridized to mRNA from M. hor-
presses DOM1, DOM2, and DOM6 mRNAs. Genomic DNA from each of these strains was subjected to PCR using primer pairs specific to DOM2, DOM4, or DOM6; the DOM2- and DOM4-specific primers are expected to direct the synthesis of 84-bp products, while the DOM6-specific primers should result in a 79-bp product (see Materials and Methods for details). The results (fig. 3) show that the DOM2 primers generated an 84-bp product with DNA from all three strains. The DOM4 primers resulted in a product with DNA from C57BL/6J and C3H/HeJ, but not with DNA from DBA/2J (fig. 3). The DOM6 primer generated product with DBA/2J DNA, but not with C57BL/6J or C3H/HeJ DNA (fig. 3). These patterns are in perfect concordance with the patterns of expression of the DOM2, DOM4, and DOM6 mRNAs in these three inbred strains (fig. 1). Thus, intraspecies variation in the expression of α1-PI mRNAs reflects the particular structural genes that are present.

### Discussion

The formation of gene families is important in the evolutionary process, since it allows the functional diversification of proteins without loss of the critical role or roles played by the "primordial" protein. In the case of SAX5, the evolution of the α1-PI structural genes within the genome is not straightforward, as new species-specific α1-PI genes are being added to the genome at an accelerated rate. This process is likely driven by the selective pressure of maintaining a functional α1-PI system in the face of changing environmental conditions. The rapid evolution of the α1-PI genes is further supported by the high degree of sequence diversity observed in the α1-PI genes across different species. This diversification is not only seen in the amino acid sequence, but also in the gene structure and regulatory elements, which suggests that the evolutionary process is ongoing and is driven by the selective pressures of the host environment.
of $\alpha_1$-PIs, extensive diversification in the reactive center has taken place following emergence of the gene family. This process appears to have been driven by positive selective forces that resulted in high levels of amino acid variability in the reactive center both among family members and among species (Hill and Hastie 1987; Borriello and Krautter 1990, 1991; Rheuame et al. 1994; Goodwin, Baumann, and Berger 1996).

Our studies have focused primarily on the $\alpha_1$-PI gene families of *M. domesticus* and *M. saxicola*. Of the 11 $\alpha_1$-PIs identified in these species thus far, five are of the orthodox type, while six are unorthodox. Four of the unorthodox inhibitors (DOM3, DOM4, DOM6, and SAX4) have tyrosine at the P1 site within the reactive center, one (DOM5) has leucine, and one (SAX5) has threonine (fig. 4). The P1 amino acid is important in determining inhibitor specificity for target serine proteases (Gettins, Patston, and Schapira 1993; Schechter et al. 1993; Potempa, Korzus, and Travis 1994). For the orthodox $\alpha_1$-PIs, the methionine residue at the P1 site appears to be required for maximal inhibition of elastase, the natural target for the inhibitor. $\alpha_1$-PIs in species with only a single gene are invariably of the orthodox type (Suzuki et al. 1991; Goto et al. 1994; Goodwin et al. 1996), reflecting the important role of orthodox inhibitors in regulating elastase activity. In contrast, species with multiple $\alpha_1$-PI genes express both orthodox and unorthodox inhibitors (Suzuki et al. 1991; Goto et al. 1994; Goodwin, Baumann, and Berger 1996). Thus, accompanying the duplication of $\alpha_1$-PI genes has been the emergence of unorthodox forms of the inhibitor, which have amino acids other than methionine at the P1 site and are likely to have physiological functions that are distinct from their orthodox relatives. For example, Saito and Sinohara (1995) have shown that three $\alpha_1$-PIs of the rabbit, each of which contains a different amino acid at the P1 position, are functionally distinct. Recent studies in our laboratory indicate that SAX1 and SAX4, which contain methionine and tyrosine, respectively, at the P1 site, exhibit differential binding to elastase (unpublished data).

Earlier studies (Hill et al. 1984; Borriello and Krautter 1990, 1991; Rheuame et al. 1994) had indicated that inbred strains of *M. domesticus* do not express the same set of $\alpha_1$-PI transcripts, prompting our interest in polymorphisms in expression of known *M. domestica* $\alpha_1$-PI mRNAs. Probes that recognize the hypervariable reactive centers and that are specific to single $\alpha_1$-PI mRNAs were utilized to demonstrate that expression of mRNAs encoding unorthodox $\alpha_1$-Pis (i.e., DOM3, DOM4, DOM5, DOM6) is highly variable among inbred strains of *M. domesticus*, while expression of mRNAs encoding orthodox $\alpha_1$-Pis (i.e., DOM1, DOM2) is relatively fixed. The strains fell into two major groups with regard to expression of unorthodox mRNAs: C57BL/6J-like strains predominantly express the DOM3, DOM4, and DOM5 mRNAs, and little or no DOM6 mRNA (fig. 1); in contrast, DBA/2J-like strains express DOM6 mRNA, with little or no DOM3, DOM4, or DOM5 mRNA (fig. 1). The variation in expression of unorthodox $\alpha_1$-PI mRNAs is likely to be genetic in origin, since the differences among inbred strains were consistently observed with RNA samples from several individual animals of each strain obtained over a 10-year period (data not shown). A similar distinction between orthodox and unorthodox $\alpha_1$-PI mRNA expression was observed in

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**Fig. 3.**—Assay for presence of specific $\alpha_1$-PI genes in *M. domesticus* inbred strains. Genomic DNAs from inbred strains C57BL/6J (B6), C3H/HeJ (C3H), and DBA/2J (DBA) were used as templates for PCR amplification of individual members of the $\alpha_1$-PI gene family, as described in Materials and Methods. For each strain, primer pairs specific for the DOM2, DOM4, and DOM6 genes (labeled as lanes 2, 4, and 6, respectively) were utilized. The 84-bp product expected with the DOM2- and DOM4-specific primers is indicated; the expected product with DOM6-specific primers is slightly shorter (79 bp).

**Fig. 4.**—The amino acid sequences of the reactive centers of the $\alpha_1$-Pis. The P1 residue is indicated by a star. Dots indicate identities relative to the sequence of the DOM1 reactive center.
the wild-derived species *M. saxoncola*. In these mice, the levels of mRNAs encoding unorthodox \( \alpha_1 \)-PIs (i.e., SAX4 and SAX5) were highly variable among individuals, while the levels of mRNAs encoding orthodox inhibitors (i.e., SAX1, SAX2, and SAX3) were not (fig. 1). Thus, extensive intraspecies variation in expression of mRNAs encoding unorthodox inhibitors may be relatively widespread in the genus *Mus*.

It is of interest to consider the role of selection in shaping the observed patterns of intraspecies variation in \( \alpha_1 \)-PI expression. The striking finding that expression of unorthodox, but not orthodox, \( \alpha_1 \)-PIs is polymorphic raises the interesting possibility that the intensity of selection may be different for the two inhibitor types. This may be related to their biochemical function(s). Perhaps the need to maintain maximal capacity to regulate elastase activity acts as a selective constraint to the emergence of variation in expression of orthodox \( \alpha_1 \)-PIs, such a constraint may not exist for the unorthodox inhibitors. Alternatively, it is possible that diversifying selective forces may actively drive high levels of polymorphism for the unorthodox \( \alpha_1 \)-PIs. If orthodox inhibitors are not susceptible to such forces, then relatively invariant expression patterns would be expected for these \( \alpha_1 \)-PIs. The nature of diversifying selective forces that may be acting on the \( \alpha_1 \)-PI family has been discussed previously (Goodwin, Baumann, and Berger 1996).

The mechanisms generating polymorphisms in \( \alpha_1 \)-PI expression are not known. The data presented in figure 3 indicate that variation in mRNA expression among strains of *M. domesticus* is due to the presence or absence of the corresponding structural genes. Thus, for example, the lack of DOM6 expression in strains C57BL/6J and C3H/HeJ reflects the absence of the DOM6 gene, rather than changes in one or more regulatory elements that modulate its transcription (fig. 3). The fact that C57BL/6J-like strains express five \( \alpha_1 \)-PI mRNAs, while DBA/2J-like strains express only three, suggests that there may be variation in \( \alpha_1 \)-PI gene copy number, where the number of expressed mRNAs reflects the size of the gene family. In this scenario, the C57BL/6J-like strains would contain five genes, while the DBA/2J-like strains would contain only three. The size of the \( \alpha_1 \)-PI family does vary among Mus species (Rheaume et al. 1994; Goodwin, Baumann, and Berger 1996), reflecting unequal crossover events that likely occurred during evolution of the mouse \( \alpha_1 \)-PI family (Goodwin, Baumann, and Berger 1996). However, it is entirely possible that DBA/2J-like strains contain genes in addition to the three already identified; these may encode novel reactive centers that are not recognized by existing probes. Clearly, a detailed analysis of the size and structure of \( \alpha_1 \)-PI genes within *M. domesticus* and *M. saxoncola* will be necessary to gain an in-depth understanding of the nature of polymorphisms in expression of this gene family.

The accumulation of amino acid substitutions in the catalytic or functional domains of proteins is relatively uncommon. Indeed, regions of functional importance are usually defined by their high degrees of conservation across taxa. The rapid evolution of the \( \alpha_1 \)-PI reactive center may have generated proteins with new or novel functional properties. Based on known biochemical functions of serpins, it has been postulated that the unorthodox \( \alpha_1 \)-PIs may have serine proteinase targets other than elastase (Saito and Sinohara 1995; Goodwin, Baumann, and Berger 1996). However, it is important to note that orthodox \( \alpha_1 \)-PI genes also exhibit rapidly diverging reactive centers (Rheaume et al. 1994; Goodwin, Baumann, and Berger 1996), indicating that changes in amino acid residues other than those at the P1 site may confer important selective advantages to the organism. Further studies may help elucidate the nature of such selective advantages and their role in sculpting the structure and function of the serpin superfamily.

In sum, studies of *Mus* \( \alpha_1 \)-PI gene families have now revealed several features that typify their evolutionary history, including gene amplification, concerted evolution, positive Darwinian selection, and regulatory alterations in expression patterns (Latimer, Berger, and Baumann 1990; Borriello and Krauter 1990, 1991; Rheaume et al. 1994; Goodwin, Baumann, and Berger 1996). Interestingly, these features also characterize changes in the mammalian lysozyme gene family during evolutionary acquisition of a digestive function in ruminants (Irwin and Wilson 1990; Irwin, Prager, and Wilson 1992; Irwin, White, and Wilson 1993; Irwin 1995). The properties shared by both the \( \alpha_1 \)-PI and lysozyme gene families may be common for small multigene families.

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Expression of the α-Proteinase Inhibitor Gene Family 427