Molecular Evolution of Aldolase A Pseudogenes in Mice: Multiple Origins, Subsequent Duplications, and Heterogeneity of Evolutionary Rates

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The Aldolase multigene family comprises three functional genes (A, B, and C) with tissue-specific expression regulated during ontogeny. DGGE analysis and nucleotide sequencing reveal a family of retropseudogenes of type A in species of Mus. Significant variation in rates of evolution of Aldolase A retropseudogenes is apparent. Our analyses demonstrate that (1) multiple events of retrotransposition are needed to account for the diversity of Aldolase A processed pseudogenes found in mice; (2) some of these sequences have undergone further duplication subsequent to the original retrotransposition event; (3) the patterns of nucleotide substitution are broadly comparable with previous estimates; and (4) estimates of rates of divergence for this array of sequences are up to four times higher than those reported in the literature.

Introduction

Sequences originating by reverse transcription from partial or completely processed messenger RNAs are called processed pseudogenes or retropseudogenes. The incorporation of retropseudogenes in the germ line is retroviral-mediated (Lee et al. 1983). Most retropseudogenes have been discovered accidentally during studies of functional genes and multigene families. In general, retropseudogenes appear to be both numerous and taxonomically widespread (Weiner 1986; Li 1997).

The lack of any apparent function and the presumed absence of selective pressures (Li, Gojobori, and Nei 1981; Kimura 1983; Li 1997) make retropseudogenes an interesting component of the genome for detailed evolutionary studies. Because all mutations occurring in a pseudogene have the same chance of being fixed in a population, they are ideal for studying patterns of spontaneous mutation and resulting biases in nucleotide composition under the assumption of neutrality (Li, Wu, and Luo 1984).

Relatively few studies have taken advantage of pseudogenes to examine patterns of substitution (and evolution or phylogenetic reconstruction) in different kinds of organisms. However, Gojobori, Li, and Graur (1982), Li, Wu, and Luo (1984), and Bulmer (1986) have documented biases in nucleotide substitutions, with an excess of changes from C to T and from G to A. Therefore, it is expected that pseudogenes will eventually become A-T-rich sequences. These trends can be interpreted as mutation biases, and we can assume in general that the deviations from the neutral evolutionary pattern are not significant (Li, Wu, and Luo 1984; Li 1997).

The present study focuses on retropseudogenes of the Aldolase A gene. Aldolase genes constitute an example of a small, dispersed multigene family. They are glycolytic enzymes associated with basal metabolism and are highly conserved in evolution. They are phylogenetically widespread, occurring in organisms as diverse as protozoans and humans (Hori et al. 1987; Kukita et al. 1988; Marsh and Lebherz 1992). In vertebrates, the gene family consists of three functional loci, A, B, and C, each apparently represented by a single copy (Sakakibara, Mukai, and Horii 1985; Tsutsumi et al. 1985; Kukita et al. 1988). Processed pseudogenes derived from Aldolase A appear to be ubiquitous in mammals. They have previously been documented in humans (Keiichiro et al. 1986) and rabbits (Amsden, Penhoet, and Tolan 1992), and recently, we amplified them in a variety of rodents, including Rattus, North American pocket gophers (Thomomys), South American tuco-tucos (Ctenomys), and long-nosed mice (Oxymycterus) (data not shown). To examine the evolutionary details of Aldolase A retropseudogenes, we identified a variety of these sequences in several species of the rodent genus Mus.

Studies of pseudogenes have often relied on comparisons of sequences obtained across divergent taxa (e.g., humans and mice), resulting in questions about the orthologous nature of the sequences and problems related to substitutional saturation. The fairly well established phylogeny of Mus and time-since-divergence estimates for several species (Bonhomme 1986; Bourjot et al. 1993; Lundrigan and Tucker 1994; Silver 1995) provide an opportunity for detailed studies of retropseudogene emergence, divergence, and overall rates and patterns of evolution.

Materials and Methods

Samples

We studied several strains of laboratory mice, as well as mice representing the species Mus musculus domesticus, Mus musculus musculus, Mus saxicola, Mus pahari, and Mus spicilegus. A complete list of individuals and their origins is shown in the appendix.

DNA Extractions and PCR Amplifications

Total genomic DNA extractions were performed using SDS–proteinase-K–sodium chloride and alcohol precipitation according to Miller, Dikes, and Polesky (1988). Primers ALD6 (5′-TGATCCTCTTCCAGGACACT-3′) and ALD11 (5′-ACGTTGCCATGGCA-
ATCTCTCTC-3′) were designed to match highly conserved sites in exons 3 and 7 of mouse Aldolase A (fig. 1). Amplifications were performed in a Thermolyne DB 66920-26 thermocycler. Fragments of approximately 530 bp, corresponding to the expected length for processed sequences (fig. 1), were obtained after 30 cycles of 95°C denaturation for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, followed by a final extension of 2 min at 72°C. Recombinant Taq polymerase from either PROMEGA or GIBCO-BRL was used. Fragments were electrophoresed on 1% agarose gels and visualized on a UV transilluminator after ethidium bromide staining.

Denaturing Gradient Gel Electrophoresis

Perpendicular denaturing gradient gels with a range of 0%–80% urea-formamide were used to determine the melting point of a representative sample and establish an optimal range for parallel denaturing gradient gel electrophoresis (DGGE; Myers, Sheffield, and Cox 1989; Lessa and Applebaum 1993). A range from 40% to 70% of denaturants was established and subsequently used to analyze PCR products obtained with primers ALD6+GC/ALD11 in parallel denaturing gels. After electrophoresis, the fragments were visualized by silver staining (Sanguinetti, Neto, and Simpson 1994).

Cloning

Two primers (ALD 6up18 [5′-GAGAUCUCUGT-GATCCTCTTCCACGAGACACT-3′] and ALD 11up18 [5′-ACGCGUACUGUACCGTCCGCTGGGAC-3′]) were modified and used for PCR of products for cloning. Amplified PCR products were excised from 1.8% agarose gels, digested overnight with Agarase I (Sigma), and cloned using the Cloning Amp TM System (Gibco-BRL). Eight to thirteen clones from each of seven selected individuals (table 1) were cycle sequenced using a 2400 Perkin Elmer thermocycler, PRISM reaction mix (Perkin Elmer), and an Applied Biosystems 377 automated sequencer. Each primer was sequenced in both directions with “Forward” and “Reverse” M13-pUC18 primers from Gibco-BRL and the ALD11 primer.

Sequencing

Eight to 13 clones from each of seven selected individuals (table 1) were cycle-sequenced using a 2400 Perkin Elmer thermocycler (initial denaturation at 96°C for 1 min, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and primer extension at 72°C for 4 min) employing PRISM Reaction Mix (Perkin Elmer) and electrophoresed on a 377 automatic sequencer (Applied Bio-Systems). Each fragment was sequenced in both directions with “Forward” and “Reverse” M13-pUC18 primers from Gibco-BRL and the ALD11 primer. Excesses of nucleotides and enzymes were removed with Centri-Sep tubes with Sephadex G-50 resin (SIGMA), centrifuged at 2,500 rpm for 2 min, and dried in a Speed Vac centrifuge under vacuum at room temperature.

Side-by-side comparisons of reamplified cloned products and the original amplicons from genomic DNA, carried out using parallel DGGE, confirmed the correspondence of the vast majority of the sequences with DGGE bands. Sequences that did not match the original bands in DGGE were discarded as likely cloning or PCR artifacts, as were redundant sequences.

Alignment and Phylogenetic Analysis

Alignment was performed with CLUSTAL W (Higgins, Bleasby, and Fuchs 1997) and optimized with...
visual inspection. Corresponding exon sequences of the functional Aldolase A genes of rat and mouse (GenBank accession numbers Y00516 and M12919, respectively) were included in all analyses. Phylogenies were obtained using equally weighted parsimony analysis in PAUP, version 4.0b2 (Swofford 1999). Distance analyses were performed using Kimura two-parameter distances and the neighbor-joining algorithm as implemented in PAUP and MEGA, version 1.01 (Kumar, Tamura, and Nei 1993). Support for nodes was assessed with 1,000 bootstrap replicas (in both distance and parsimony analyses).

Substitution biases were assessed as follows. First, the topology of the neighbor-joining tree, excluding the functional genes, was input into MacClade, version 3.07 (Maddison and Maddison 1997). The program estimated the average number of substitutions of each type that must have taken place along the branches of the tree using the parsimony criterion. To obtain relative rates of substitutions, these values were divided by the frequency of each nucleotide. These were estimated for all data, as well as exclusively for the mouse and rat functional genes, and the results were essentially identical. Finally, a separate analysis was conducted using the same methods but excluding CG dinucleotides to eliminate the possible effects of these dinucleotides on C-to-T changes (Gojobori, Li, and Graur 1982; Li, Wu, and Luo 1984; see Results and Discussion).

Evolutionary rates were estimated by two methods. First, pairwise distances (Kimura two-parameter model) between sequences were estimated and plotted against estimated times of divergence of the species taken from Boursot et al. (1993) and Silver (1995). This was done independently for each of the major clades identified in the phylogeny. Intraspécific comparisons within M. musculus domesticus were assigned a maximum age of 0.5 Myr, as suggested by Silver (1995). A second method entailed the computation of maximum-likelihood estimates of branch lengths (substitutions along branches) of the neighbor-joining topology as implemented in PAUP 4.0b2 (Swofford 1999). Unlike pairwise comparisons, maximum-likelihood estimates are deemed independent of each other. However, they demand more assumptions about the nature of evolutionary changes and about the correctness of the tree. We also conducted a likelihood ratio test of the molecular clock for all pseudogenes by comparing a model that assumes a single underlying rate of evolution with one that releases that constraint and allows variation in rates among branches (Felsenstein 1995b).

Results and Discussion
DGGE Analysis

PCR products obtained from a total of 43 individuals examined in agarose gels revealed bands of about the expected size, i.e., about 530 bp for each individual (table 1 and fig. 2a). However, direct sequencing of such PCR products resulted in overlapping sequences, suggesting the presence of multiple amplicons. Perpendicular DGGE assays showed many sigmoid curves, thus confirming that multiple fragments had been coamplified (data not shown). Parallel DGGE allowed discrimination of these multiple products.

Several bands appeared to be shared across species, whereas others varied among species or even among conspecific individuals. For example, figure 2b shows variation among M. musculus domesticus from two Uruguayan localities, “La Paz” and “El Relincho,” in which a basic pattern of four bands with different migratory positions is observed. In figure 2c, variation among M. musculus domesticus, M. pahari, and M. spicilegus is shown.

Computational (Lerman and Silverstein 1985) and empirical studies of DNA fragments differing by single-base-pair substitutions in correspondence with melting theory have been carried out (Fixman and Freire 1977; Fisher and Lerman 1983). Changes in migration during DGGE are predictable for single mutations but not for multiple substitutions as in our case. Thus, bands with different migratory behaviors must differ in sequence, but the converse is not necessarily true for a complex set of amplicons.

The number and pattern of observed bands suggest the presence of more than one locus (of processed fragments) in all cases. Thus, at least nine distinct bands are observed in M. pahari (fig. 2c), which must therefore have at least five distinct loci. Even among individuals of the same population, it was possible to detect different bands, suggesting intrapopulational, possibly allelic, variation. In sum, DGGE analysis suggests a minimum of three or possibly four loci.

Phylogenetic Analysis of Sequence Variation

To corroborate and further examine the rich diversity of retropseudogenes suggested by DGGE, a subset of PCR products from seven individuals spanning the range of taxonomic diversity (table 1) was subjected to multiple cloning and sequencing (see Materials and Methods) to produce a total of 43 distinct sequences. Variation among processed amplicons ranged from single substitutions to 30% uncorrected sequence divergence, with typical values of about 10%. The presence of multiple insertions and deletions confirmed the pseudogene status of these sequences.

Processed pseudogenes may originate by reverse transcription from mature mRNA and subsequent insertion in the genome. Once one of such processed pseudogenes has been formed, additional copies may be generated by subsequent duplications. Each of these two pseudogene-generating mechanisms—reverse transcription and subsequent duplication—leaves a characteristic phylogenetic pattern (fig. 3). A phylogeny consisting solely of independently generated retrocopies from a single individual should be starlike (Felsenstein 1995a); i.e., it should show no demonstrable cladistic relationships among loci other than through their historical connections with the functional gene. In contrast, pseudogenes generated by duplication are no different from other gene families in that paralogous loci are related to...
Evolution of Aldolase A Pseudogenes in Mice

Fig. 3.—Processes resulting in the proliferation of processed pseudogenes and resulting phylogenetic patterns for sequences obtained from a single individual. a. Processed pseudogenes produced by insertion in the genome of independently generated retrocopies; substitutions are autapomorphies, and the corresponding phylogeny is an unresolved polytomy. b. Pseudogenes generated by subsequent duplication of a single retrocopy; phylogenetic relationships among pseudogenes are supported by synapomorphies.

Each other by a dichotomous gene tree that reflects the sequence of duplications (fig. 3).

A phylogeny of Aldolase A retrocopies was generated in order to gain insights into the possible sources of pseudogene diversity. Only minor variation in the resultant gene tree was observed when different distance estimates were used in the neighbor-joining analysis or between these and maximum parsimony (not shown), so the discussion will be based on a neighbor-joining tree using Kimura two-parameter distances.

The neighbor-joining tree (fig. 4) combines the two patterns outlined above (fig. 3) and therefore suggests that several loci were originated by independent insertion of reverse transcripts and that in some cases such inserts underwent subsequent duplications. Thus, there are four strongly supported clusters (A–D in fig. 4) of pseudogene sequences that connect to the base of the phylogeny with the mouse and rat functional genes. These four clusters likely represent independently generated retrocopies. However, there is also evidence for the presence of more than one locus in some of these clusters, suggesting that additional loci were generated by pseudogene duplication. These two features of our phylogenetic tree—independent retrotransposition and subsequent duplication—are illustrated by the following features of the phylogeny:

1. Cluster A shows strong internal phylogenetic structure, which in turn suggests two or three events of duplication after the original retrotransposition. For example, M. spicilegus is represented by four distinct sequences in this cluster, only two of which are likely alleles of a single locus.
2. Cluster B shows two distinct lineages resulting from a duplication of the original retropseudogene. One of them is found exclusively in M. pahari. The second lineage is represented in all species, including M. pahari. Interestingly, this second lineage, which is best interpreted as a single locus, mirrors the accepted phylogeny of the genus Mus (Boursot et al. 1993; Silver 1995), as shown in figure 5.
3. Cluster C is found exclusively in M. pahari. Here, as elsewhere, the absence of lineages in certain species may be the result of (1) species-specific events of retrotransposition or duplication, (2) loss of pseudogenes in certain species, and (3) effects of sampling in PCR and cloning.
4. Cluster D is exceptional in that it contains four to five sequences from each representative of M. musculus domesticus. These represent up to 80% of the sequences recovered from each individual for this species. Furthermore, although the data indicate the presence of at least two loci produced by duplication...
of an original retropseudogene, the variants are all extremely similar. Unlike other clusters, it is impossible to offer a clear delineation of loci. These observations suggest that one or more very recent events of duplication have taken place in *M. musculus domesticus*.

Substitution Patterns and Compositional Bias

It has been demonstrated that nucleotide substitutions are not random. The pattern inferred by Gojobori, Li, and Graur (1982) and Li (1997) is an estimate of the mutation bias in mammalian nuclear DNA, where tran-
Estimates of nucleotide substitutions reconstructed with MacClade (Maddison and Maddison 1997) on the phylogeny (fig. 4) show clear biases in favor of transitions over transversions. Among the former, C-to-T changes are the most frequent. Indeed, estimation of nucleotide substitutions excluding CG dinucleotides essentially eliminates the excess of C-to-T over A-to-G transitions (fig. 6). More generally, the patterns of nucleotide substitutions in aldolase pseudogenes compare well with those previously estimated by O‘hUigin and Li (1992). A comparison of the average nucleotide composition in our collection of pseudogenes with the corresponding regions of the functional Aldolase A gene of Mus shows no significant difference ($\chi^2 = 1.332, P > 0.72$). It seems that there has not been enough time for the observed substitution biases to result in significant changes in nucleotide frequencies toward A-T.

It has been established that deletions are about twice as frequent as insertions for nuclear DNA in comparisons between processed pseudogene sequences of humans and rodents (Graur, Shuali, and Li 1989), and the same tendency has been found by analyzing non-coding regions of primates (Saitou and Ueda 1994). In contrast, no obvious trend toward insertions or deletions was observed in our data. Using parsimony, we estimated that at least 13 insertions and 16 deletions, mostly affecting one or a few nucleotides, must have occurred in the history of Aldolase A pseudogenes (details not shown). These estimates, however, cannot evaluate the occurrence of large-scale insertions or deletions, whose remnants cannot be recovered in our sample.

Molecular Clock and Rates of Evolution

Figure 7 plots pairwise Kimura two-parameter distances against estimated times of divergence between species of Mus (Boursot et al. 1993; Silver 1995). Values for each of the major clusters in the phylogeny fit different curves, suggesting rate variation among them. Furthermore, our slopes suggest rates of pseudogene evolution that are two- to fourfold larger than those estimated by O‘hUigin and Li (1992).

There are several possible reasons for the observed discrepancies, both among aldolase pseudogene clusters and between these and previous estimates. First, all of these estimates are subject to considerable sampling errors because pairwise distances are not independent observations. Second, we compared a large number of closely related sequences, all belonging to the genus Mus, whereas previous estimates relied on comparisons between genera and higher taxa (typically Mus, Rattus, and humans; see Li, Wu, and Luo 1985; O‘hUigin and Li 1992). The latter may therefore underestimate actual rates of substitutions because of saturation in spite of the use of distance values that include corrections for such effects. Finally, there may be actual variation in rates across different genomic regions and species.

A likelihood ratio test yields support for the latter alternative. We computed the best maximum-likelihood tree for the aldolase pseudogenes, excluding the functional genes, using a 2:1 rate of transitions over transversions and no enforcement of a molecular clock. The resulting topology and a similar transition : transversion bias were used in a second model that enforced a molecular clock. As suggested by Felsenstein (1995b), these two models may be statistically compared to produce a test of the molecular-clock hypothesis. In this case, the model without a clock is significantly better ($-2 \ln(L) = 2.09523500, \chi^2 = 51.61505, P < 0.001$). This analysis does not eliminate the possible effects of
Fig. 6.—Patterns of nucleotide substitutions in Aldolase A pseudogenes compared with relative-rate estimates provided by Li and Graur (1991). 

(a) Base composition estimated from the entire aldolase data set. 
(b) Base composition estimated exclusively from rat and mouse functional genes. In both cases, CG dinucleotides were excluded from the data.

Different times of origin of the four major clusters. Nonetheless, it reinforces the idea of substantial rate heterogeneity among aldolase pseudogenes. It should come as no surprise that, in turn, these data show somewhat different rates than published estimates.

Conclusions

DGGE studies and posterior sequencing of PCR cloned fragments have revealed an extensive multigene family of processed Aldolase A pseudogenes in mice. Although pseudogenes are very common within multigene families, their levels of production and survival are very different depending on the groups examined. They appear to be abundant in mammals but relatively rare in other organisms such as chickens, amphibians, and Drosophila (Li 1997).

Our family of at least eight pseudogene loci, derived from a single functional gene, is one of the largest described thus far (Weiner 1986; Dhawan et al. 1998). We have found evidence of both independent retrotranscription and subsequent duplication being sources of pseudogene diversity. The underlying process responsible for the generation of pseudogenes deserves further analysis. One explanation for the abundance of pseudogenes may relate to a very active expression rate in germ line tissues, facilitating the generation of retrocopies (Lee et al. 1983; Lindsey and Wilkinson 1996a, 1996b, 1996c; Maiti et al. 1996). In some cases, however, positive selection may be responsible (Sutton and Wilkinson 1997).

A related, unresolved question is whether there is any association between the amount of retropseudosequences generated and the location of their parental functional genes within early- or late-replicating chromatin. Alternatively, there could be a relationship between the stability of the functional genes’ mRNAs and this high number of retrocopies.

If the chance of fixation depends on the expression level in the germ line, two other predictions should hold: (1) other genes from families with high levels of expression in the germ line should have incorporated retrosequences; and (2) males, with continuous production of sperm, should have more suitable conditions in the germ line for the accumulation of retrocopies in comparison with females with a number of reproductive cells fixed, arrested in meiotic prophase (Lee et al. 1983). It is just as important to examine what triggers the secondary duplication of retropseudogenes.

Because of their relatively rapid, and presumably neutral, evolution, processed pseudogenes are in principle ideal for phylogenetic analysis of species relationships. However, the diversity, multiple origins, and complex history of Aldolase A pseudogenes indicate that molecular phylogeneticists should take great care in distinguishing orthologous from paralogous sequences. Although we cannot be sure at this point, it is quite possible that families of related pseudogenes such as the one uncovered here are very common (Weiner 1986).
Supplementary Material

All pseudogene sequences found were deposited in GenBank under accession numbers AYO15228–AYO15270.

Acknowledgments

We are grateful to Wen-Hsiung Li, Matthew P. Hare, Rodney Honeycutt, and two anonymous reviewers for offering valuable comments and suggestions. We thank Ellen Prager, Mario Clara, and Toti Altuna for furnishing tissues and DNA of mice, as mentioned in Materials and Methods; Joseph Cook and his lab crew at the University of Alaska, Fairbanks, where automated sequences were generated; and Nella, Lucía, Felipe, and Tomás Cook for letting M.N.C. be part of their family for a month. We also thank the members of Secciones Bioquímica, Genética y Fisiología Bacterianas, and Genética Evolutiva for their assistance, especially when our laboratory was still in the installation period. We are indebted to Federico Hoffmann, Guillermo D’Elóa, Alejandra Chiesa, Ivanna Tomasco, and Gabriela Wlasiuk, our workmates in the laboratory, for their daily support. Financial assistance was provided by PEDECIBA (Programa para el Desarrollo de las Ciencias Básicas), CSIC (Comisión Sectorial de Investigación Científica, Universidad de la República), and CONICYT (Consejo Nacional de Investigaciones Científicas y Técnicas), Uruguay.

LITERATURE CITED

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WILLIAM TAYLOR, reviewing editor

Accepted May 11, 2001
APPENDIX
Description of the Sample

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<td>42</td>
<td>M1019</td>
<td>M. musculus domesticus</td>
<td>El Relincho, Departamento San José, Uruguay</td>
</tr>
<tr>
<td>43</td>
<td>M1029</td>
<td>M. musculus domesticus</td>
<td>Ciudad Vieja, Montevideo, Uruguay</td>
</tr>
</tbody>
</table>

Note.—List of studied individuals by DGGE and their origins. Each individual is identified with “M.” indicative of mice, followed by the number recorded in EV and MC catalogs at Facultad de Ciencias, Montevideo, Uruguay.