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Evolutionary Story of Mammalian-specific Amelogenin Exons 4, “4b”, 8, and 9

ABSTRACT

Amelogenin gene organization varies from 6 exons (1,2,3,5,6,7) in amphibians and sauropsids to 10 in rodents. The additional exons are exons 4, 8, 9, and “4b”, the latter being as yet unidentified in *AMELX* transcripts. To learn more about the evolutionary origin of these exons, we used an *in silico* approach to find them in 39 tetrapod genomes. *AMEL* organization with 6 exons was the ancestral condition. Exon 4 was created in an ancestral therian (marsupials + placentals), then exon 9 in an ancestral placental, and finally exons “4b” and 8 in rodents, after divergence of the squirrel lineage. These exons were either inactivated in some lineages or remained functional: Exon 4 is functional from artiodactyls onward; exon 9 is known, to date, only in rodents, but could be coding in various mammals; and exon “4b” was probably coding in some rodents. We performed PCR of cDNA isolated from mouse and human tooth buds to identify the presence of these transcripts. A sequence analogous to exon “4b”, and to exon 9, could not be amplified from the respective tooth cDNA, indicating that even though sequences similar to these exons are present, they are not transcribed in these species.

KEY WORDS: amelogenin, small exons, evolutionary origin, PCR, enamel, tetrapods.

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A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

INTRODUCTION

Amelogenin, the major protein of forming enamel, mainly plays a role in crystal growth (Robinson *et al*., 1996; Beniash *et al*., 2005). Its encoding gene (*AMELX* = *AMEL* in non-mammalian species) is composed of 7 exons in mammals, except in rats and mice, in which 2 additional exons (8 and 9) have been found (R Li *et al*., 1995; W Li *et al*., 1998). These exons and exon 4 are not present in non-mammalian *AMEL*. *AMELX* is subjected to alternative splicing, giving rise to several transcripts and various isoforms. Some of them might possess signaling capabilities. In the mouse, 17 *AMELX* transcripts have been identified, among which 7 lack exon 7 and end with exons 8 and 9 (R Li *et al*., 1995; W Li *et al*., 1998; Bartlett *et al*., 2006). In 2006, when analyzing the genomic region containing *AMELX* exon 8, Bartlett and colleagues revealed that this exon was homologous to exon 5. In addition, they found that a small sequence located immediately upstream of exon 8 was identical to the exon 4 sequence, and they referred to this as a putative exon 4b. Exons 4b and 8 were, therefore, generated from the duplication of a gDNA segment containing exons 4 and 5, which was translocated downstream of exon 7. Surprisingly, exon 4 was never found in *AMELX* transcripts identified in rodent cDNA, and hence hereafter are marked “4b”.

Using *in silico* investigations, we traced the origin of mammalian-specific *AMELX* exons 4, “4b”, 8, and 9 through tetrapod evolution. Our analyses provide information on the birth of these exons and led us to wonder whether *AMELX* exon “4b” is coding in the mouse, and whether exon 9 is present in human *AMELX* and *AMELY* transcripts. We addressed these questions using PCR.

MATERIALS & METHODS

In silico Searches

In total, 39 sequenced tetrapod genomes [37 mammals, a lizard (*Anolis carolinensis*), and a frog (*Xenopus tropicalis*)] were explored for *AMEL* exons 4, “4b”, 8, and 9. Published sequences were used as a template for localization of the sequences in the genomes by BLAST. The regions potentially housing *AMEL* exon 4, 1.5 kb between exons 3 and 5, and *AMEL* exons “4b”, 8, and 9, 20 kb downstream of exon 7, were extracted from each genome (Fig. 1). These regions were screened with UniDPlot (Girondot and Sire, 2010), with human and rodent sequences of the targeted exons as a template. The sequences were validated by means of alignment with human and murine sequences with Se-Al 2.0 (Rambaut, 1996).

References of the studied genomes and *AMEL* sequences are listed in Appendix 1.

Selective Pressure Analysis by the Hyphy Method

Hyphy software (http://hyphy.org; Pond *et al*., 2005) was used in the search for selective pressures that acted on exon 4 during evolution (Appendix 2).

PCR

Primers were designed with Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) (Appendix 3). PCRs were performed on mouse genomic DNA (gDNA) with primer pair M1, to obtain an accurate sequence of the non-coding genomic region located between exons 7 and 8. This aimed to check whether *AMELX* exon "4b" sequence was really present in the mouse genome or was an artifact resulting from an incorrect computer-predicted sequence assembly. PCRs were also done on a mouse tooth bud cDNA library with primer pairs M2, M3, and M4, to find transcripts possessing the putative exon "4b". In humans, utilizing primer pairs H1 and H2, we performed PCR on cDNAs for putative transcripts ending with exon 9 (including a human fetal tooth cDNA library and cDNA samples freshly prepared from fetal human tooth buds, collected under the guidelines of the University of California Committee on Human Research), by using the RNeasy Mini Kit and SuperScript III Reverse Transcriptase Kit. Both in humans and mice, the primers designed for PCRs on cDNA were used to amplify gDNA, to demonstrate the effectiveness of the primer sets.

RESULTS

AMELX Exon 4

Exon 4 was found in all mammalian genomes, except in the monotreme platypus (Fig. 2). Exon 4 is absent in lizard and frog gDNA. In several mammals [a primate (marmoset) out of 11 species studied, 7 laurasiatherians out of 11, the 2 xenarthrans, the 3 afrotherians, and the 2 marsupials], our analysis indicated that the putative exon 4 was inactivated (see Fig. 2). In contrast, it is possibly coding in primates, the tree shrew, rodents, artiodactyls (cow, alpaca, and pig), the cat, and in human *AMELY,* in which it possesses correct intronic splice sites and no deleterious mutation (Fig. 2). Selective pressure analysis identified the second residue encoded by exon 4 as being negatively selected (*i.e*., conserved) and residues 3, 7, 8, and 13, and the intronic splicing site as being positively selected (see Appendix 2).

AMELX Exon "4b"

In the mouse, sequencing the 2.0-kb genomic region separating exons 7 and 8 provided a sequence identical (not shown) to that available in databases, which means that the gDNA sequence containing exon "4b", and identical to the sequence around exon 4, is a valid sequence and not an artifact generated during computer-aided assembly of this region.

A search for exon "4b" downstream of *AMEL* exon 7 in all genomic sequences revealed that a homologous sequence is present in murids (mouse, rat, and the 2 related species) and in caviids (guinea pig), while it is absent in sciurids (squirrel) (Fig. 3A). It could be coding in the guinea pig, rat, deer mouse, and mouse, while not coding in the kangaroo rat, in which the splice acceptor site is mutated. The inactivating mutation of exon "4b" in this species could have favored the accumulation of numerous substitutions, as observed in its sequence.

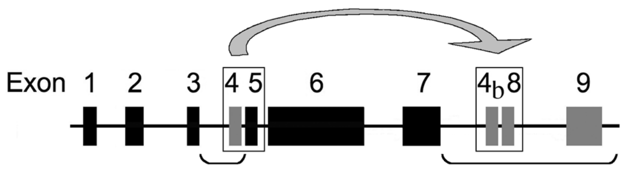


Figure 1. *AMELX* organization in the mouse genome showing the 10 exons: exons 1 to 9 and the putative exon 4b. Exon 4b and exon 8 were created after the DNA region containing exon 4 and exon 5 was duplicated and translocated downstream of exon 7 (arrow). The regions that were explored in various genomes for exons 4, 4b, 8, and 9 (gray blocks) are indicated with brackets.

However, we failed to PCR amplify exon "4b" from a mouse tooth bud cDNA library.

AMELX Exon 8

Genome exploration downstream of *AMEL* exon 7 revealed that exon 8 is present, in addition to the mouse and rat, in only 3 other rodent *AMELX* (Fig. 3B). It was not found in the squirrel and in all non-rodent species, even as pseudogenic. Sequence analysis pointed to several substitutions when compared with the sequences of exon 5, from which it is derived. However, with the intronic splice sites being correct and no deleterious mutation being observed, there is no reason to think that *AMELX* exon 8 was not coding in the deer mouse, kangaroo rat, and guinea pig (Fig. 3B).

AMELX Exon 9

Screening the region downstream *AMEL* exon 8 in all genomes not only revealed the presence of exon 9 in all rodent sequences possessing exon 8, but also demonstrated the presence of a sequence with a high percentage of nucleotide identity in all placental *AMELX*, including human *AMELX* and *AMELY*. No sequence similarity was found in marsupial, monotreme, and non-mammalian gDNA.

Alignment of all putative exon 9 against murine sequences showed that most sequences, including human *AMELX* and *AMELY* exon 9, possessed a correct intron splice donor site at their 5′ side, along with a stop codon (Fig. 3C). In a few sequences only (*e.g.,* kangaroo rat), the putative intron splice site is mutated, which indicates either that exon 9 is not coding (*i.e.,* independently inactivated in a few species) or that another putative intron splice site is located upstream but is hard to define. The sequence length of exon 9 is variable (*e.g*., 15 bp in guinea pig, 27 in mouse, 60 in human *AMELX,* but 27 in *AMELY*, 69 in shrew), but the nucleotide identity in homologous regions indicates that this exon is probably coding. A putative polyadenylation signal is also

```
                                           [                            exon 4                            ]
                HumanX               TAG   AAC--TCA CAT TCT CAG GCT ATC AAT GTT GAC AG----G ACT GCA---TTA   GTG
                HumanY               TAG   AAC--TCA CAT TCT CAG GCT ATC AAT GTT GAC AG----G ATT GCT---TTA   GTG
                Chimpanzee           TAG   AAC--TCA CAT TCT CAG GCT ATC AAT GTT GAC AG----G ACT GCA---TTA   GTG
                Gorilla              TAG   AAC--TCA CAT TCT CAG GCT ATC AAT GTT GAC AG----G ACT GCA---TTA   GTG
                Orangutan            TAG   AAC--TCA CAT TCT CAG GCT ATC AAT GTT GAC AG----G ACT GCA---TTA   GTG
                Gibbon               TAG   AAC--TCA CAT TCT CAG GCT ATC AAT GTT GAC AG----G ACT ACA---TTA   GTG
Primates        Rhesus monkey        TAG   AAC--TCA CAT TCT CAG GCT ATC AAT GTT GAC AG----G ACT GCA---TTA   GTG
                Baboon               TAG   AAC--TCA CAT TCT CAG GCT ATC AAT GTT GAC AG----G ACT GCA---TTA   GTG
                Squirrel monkey      TAG   AAC--TCA CAT TCT CAG GCT ATT AAT ATT GAC AG----G ACT GGA---TTA   GTG
                Ring-tailed lemur    TAG   AAC--TCA CAT TCT CAG GCT ATC AGT ATT TAC GG----G ACT GAA---TTA   GTG
                Marmoset           # TAG   AAC--TCA --T TCT CAG GCT ATC AAT ATT GAC AG----G ACT GGA---TTA   GTG
                Bushbaby             TAG   AAG--TCA TAT TCT CAG GCT ATC AAT ATT GAC AG----G ACT GCA---TTA   GTG
                Mouse lemur          TAG   AAC--TCA CAT TCT CAG GCT ATC AAT ACA CAC AG----G ACT GAA---TTA   GTG
                Tarsier              TAG   AAC--ACA TAT TCT CAG GCT ATC AGT ATT GAC AG----G ACT GCA---TTA   GTG
                Tree shrew           TAG   AAC--TCA CAT TCT CAG GCT ATC AAT ATT GGC AG----G ACT GCA---TTA   GTG
                Mouse                TAG   AAG--TCA CAT TCT CAG GCT ATC AAT ACT GAC AG----G ACT GCA---TTA   GTG
                Deer mouse           TAG   AAG--TCA CAT TCT CAG GCT ATC AAT ACT GAC AG----G ACT GCA---TTA   GTG
Rodentia        Rat                  TAG   AAG--TCA CAT TCT CAG GCT ATC AAT ACT GAC AG----G ACT GCA---TTA   GTG
                Kangaroo rat         TAG   AAG--TCA CAT TCT CAG GCT ATC AAT ACT GAC AG----G ACT GCA---TTA   GTG
                Guinea pig           TAG   AAG--TCA CAT TCT AAC GCT ATC AAT ATT GAC AG----G ACT GCA---TTA   GTG
                Squirrel             TAG   AAA--TCA CAT TCT AAG GCT ATC AAT ACT GAC AG----G ACT GCA---TTA   GTG
                Cow                  TAG   AAC--TCC TAT TTT CAG GGT ATC AGT ATT GAC GA----G ACT GCA---TTA   GTG
                Alpaca               TAG   AAC--TCA TAT TTT CAG GCT ATC AGT ATT GAC AG----G ACT GCA---TTA   GTG
                Pig                  TAG   GAC--TTA TAC TTG GAG GCT ATC CGT ATT GAC AG----G ACT GCA---TTC   GTG
                Dolphin            # TAG   AGC----A TAT TTT CAG GTT GTC CGT ATT GAC GG----G ACT GCA---TCA   GTG
                Horse              # TAG   AAC--TCG TAT TAT CAG GCT ATC AGT ATT GAC AGTATTG ACT GCA---TTA   GCG
Leurasiatheria  Dog                # CAG   GTG---CC C-C TT- -GG GCT ATC AAT ATT GAC AG----G ACT GCA---TCA   GTG
                Cat                  TAG   AAC--TCA CAC TTT CAG GCT ATC GAT ATT GAC AG----G ACT GCAGCATCA   GTG
                Macrobat           # TAG   AAC--TTC TAT TTT CAG GCT ATG AAT ATT GGC AG----G ACT GCA---TTA   TTG
                Microbat           # TAG   AAC--TCA TAT TTT TAG GTT ATC AAT GTT GAC AG----G ACT GCA---TTT   TTG
                Hedgehog           # TAG   AAC--TCC TAC TTT CAA GCT ACC AAT ACT GAC AG----G ACT CCA---TTA   GCA
                Shrew              # TAG   AACTATCA GGC TAT CAG TCT ATC ATG ATT GAT GG----G GTT ACA---TTA   TTG
Xen             Armadillo          # TAG   ACC--TCA CAT TTT CAG GCT ATC ACA ATT GAC AA----G ACT GCA---TTA   ATG
                Sloth              # TAG   ATC--TCA TGA TTT CCA GCT ATT GCT ATC GAC AA----G ACT GCA---TTA   GTG
                Elephant           # GTA   AAG--TCA CAA TTT CAG GCT ACC AGT ATT GAC AG----G ACT GCA---GGA   GGG
Afr             Tenrec             # CAG   AAG--TCA CAG CTT CAG GCT AAC GCT AAT AGG CT----G GGG GTG---GGG   GAG
                Hyrax              # GTC   AAG--TCA CAA TTT CAG GCT ACC AAT ACT GAC AG----G ACT GCA---GGA   GTG
Mar             Wallaby            # TAG   AGC--TCA AAC TTA ATC GCT ATA AAT GTT GAA AT----G ACT TTC---ATT   GAG
                Opossum            # CTG   GAA--ACA AAC TTA TTT GCA AGG AGT ATT --- ------A ACT GGA---TTA   TTT
                                                 -   +                   +   +                         +     +
```

Figure 2. Alignment of 37 nucleotide sequences of either functional (*i.e.,* found in cDNA, species indicated in bold), or putatively functional (but no cDNA data), or inactivated (see below) *AMELX* exon 4 recovered in the genome of representative species of mammalian lineages. Human *AMELY* exon 4 was included in this alignment; it exhibits only 2 nucleotide substitutions (underlined) and looks functional. The important nucleotides of the donor (left) and acceptor (right) intron splices are indicated on both sides of the alignment. Exon 4 is assumed to be functional in all primates except in the marmoset, in the tree shrew, in rodents, and in a few laurasiatherians. In contrast, exon 4 is inactivated (# = pseudogenic) in numerous lineages; it shows either splice-site-mutated (in gray background) or deleterious mutations (reading frame shift or stop codon, underlined). Selective pressure analysis (Hyphy method, see Appendix 2) identified 1 negatively (-) and 5 positively (+) selected sites. Latin names of species and accession numbers of sequences are indicated in Appendix 1. Afr = afrotherians; Mar = marsupials; Xen = xenarthrans.

at the correct location as compared with rodent sequences (not shown). Given the similarity between *AMELX* exon 9 sequence in representatives of most placental mammal lineages, *i.e.,* covering a period of 104 million years (Ma) of evolution (Fig. 4), we believe that exon 9 is coding; otherwise, mutations would have accumulated at random.

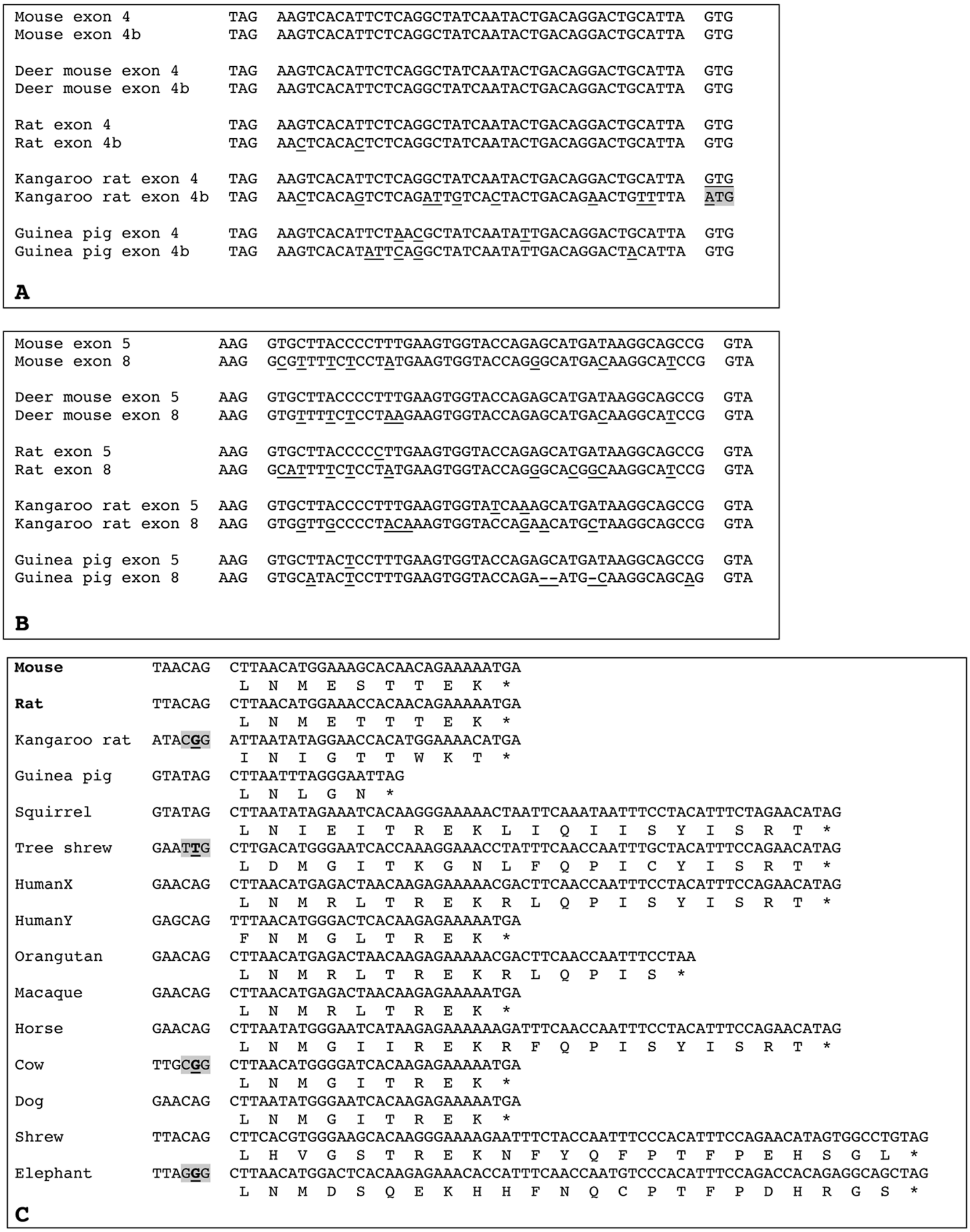
However, we have not been able to PCR amplify exon 9 from human fetal tooth cDNA.

DISCUSSION

AMELX Exons 4, "4b", 8, and 9 are Mammalian Innovations

The exploration of 39 tetrapod genomes allowed us to trace the origin of *AMEL* exons 4, "4b", 8, and 9 in tetrapod evolution and to demonstrate that they are mammalian-specific *AMELX* exons. Indeed, these exons are not present in lizard and frog gDNA, confirming previous published *AMEL* transcript sequences in reptiles (Ishiyama *et al*., 1998; Delgado *et al*., 2006; Wang *et al*., 2006) and amphibians (Toyosawa *et al*., 1998; Diekwisch *et al*., 2009). None of these exons was found in the platypus genome, confirming previously sequenced *AMEL* transcripts (Toyosawa *et al.*, 1998). This means that *AMELX* was composed of 6 functional exons (1-3, 5-7) in the last common ancestor of therian mammal (placentals + marsupials), *i.e., circa* 176 Ma ago.

Exon 4 appeared in an ancestral therian, *i.e.*, between 220 and 176 Ma ago, but was not functional, confirming previous cDNA sequencing in the opossum (Hu *et al*., 1996). It was retained as a functional exon only later in placental evolution (thus, 7 exons for *AMELX*). Then, exon 9 was recruited in an ancestral placental, but was not retained in several lineages.



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Mouse exon 4          TAG  AAGTCACATTCTCAGGCTATCAATACTGACAGGACTGCATTA  GTG
Mouse exon 4b         TAG  AAGTCACATTCTCAGGCTATCAATACTGACAGGACTGCATTA  GTG

Deer mouse exon 4     TAG  AAGTCACATTCTCAGGCTATCAATACTGACAGGACTGCATTA  GTG
Deer mouse exon 4b    TAG  AAGTCACATTCTCAGGCTATCAATACTGACAGGACTGCATTA  GTG

Rat exon 4            TAG  AAGTCACATTCTCAGGCTATCAATACTGACAGGACTGCATTA  GTG
Rat exon 4b           TAG  AACTCACACTCTCAGGCTATCAATACTGACAGGACTGCATTA  GTG

Kangaroo rat exon 4   TAG  AAGTCACATTCTCAGGCTATCAATACTGACAGGACTGCATTA  GTG
Kangaroo rat exon 4b  TAG  AACTCACAGTCTCAGATTGTCACTACTGACAGAACTGTTTTA  ATG

Guinea pig exon 4     TAG  AAGTCACATTCTAACGCTATCAATATTGACAGGACTGCATTA  GTG
Guinea pig exon 4b    TAG  AAGTCACATATTCAGGCTATCAATATTGACAGGACTACATTA  GTG

A
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Mouse exon 5         AAG  GTGCTTACCCCTTTGAAGTGGTACCAGAGCATGATAAGGCAGCCG  GTA
Mouse exon 8         AAG  GCGTTTTCTCCTATGAAGTGGTACCAGGGCATGACAAGGCATCCG  GTA

Deer mouse exon 5    AAG  GTGCTTACCCCTTTGAAGTGGTACCAGAGCATGATAAGGCAGCCG  GTA
Deer mouse exon 8    AAG  GTGTTTTCTCCTAAGAAGTGGTACCAGAGCATGACAAGGCATCCG  GTA

Rat exon 5           AAG  GTGCTTACCCCCTTGAAGTGGTACCAGAGCATGATAAGGCAGCCG  GTA
Rat exon 8           AAG  GCATTTTCTCCTATGAAGTGGTACCAGGGCACGGCAAGGCATCCG  GTA

Kangaroo rat exon 5  AAG  GTGCTTACCCCTTTGAAGTGGTATCAAAGCATGATAAGGCAGCCG  GTA
Kangaroo rat exon 8  AAG  GTGGTTGCCCCTACAAAGTGGTACCAGAACATGCTAAGGCAGCCG  GTA

Guinea pig exon 5    AAG  GTGCTTACTCCTTTGAAGTGGTACCAGAGCATGATAAGGCAGCCG  GTA
Guinea pig exon 8    AAG  GTGCATACTCCTTTGAAGTGGTACCAGA--ATG-CAAGGCAGCAG  GTA

B
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```
Mouse         TAACAG  CTTAACATGGAAAGCACAACAGAAAAATGA
                       L  N  M  E  S  T  T  E  K  *
Rat           TTACAG  CTTAACATGGAAACCACAACAGAAAAATGA
                       L  N  M  E  T  T  T  E  K  *
Kangaroo rat  ATACGG  ATTAATATAGGAACCACATGGAAAACATGA
                       I  N  I  G  T  T  W  K  T  *
Guinea pig    GTATAG  CTTAATTTAGGGAATTAG
                       L  N  L  G  N  *
Squirrel      GTATAG  CTTAATATAGAAATCACAAGGGAAAAACTAATTCAAATAATTTCCTACATTTCTAGAACATAG
                       L  N  I  E  I  T  R  E  K  L  I  Q  I  I  S  Y  I  S  R  T  *
Tree shrew    GAATTG  CTTGACATGGGAATCACCAAAGGAAACCTATTTCAACCAATTTGCTACATTTCCAGAACATAG
                       L  D  M  G  I  T  K  G  N  L  F  Q  P  I  C  Y  I  S  R  T  *
HumanX        GAACAG  CTTAACATGAGACTAACAAGAGAAAAACGACTTCAACCAATTTCCTACATTTCCAGAACATAG
                       L  N  M  R  L  T  R  E  K  R  L  Q  P  I  S  Y  I  S  R  T  *
HumanY        GAGCAG  TTTAACATGGGACTCACAAGAGAAAAATGA
                       F  N  M  G  L  T  R  E  K  *
Orangutan     GAACAG  CTTAACATGAGACTAACAAGAGAAAAACGACTTCAACCAATTTCCTAA
                       L  N  M  R  L  T  R  E  K  R  L  Q  P  I  S  *
Macaque       GAACAG  CTTAACATGAGACTAACAAGAGAAAAATGA
                       L  N  M  R  L  T  R  E  K  *
Horse         GAACAG  CTTAATATGGGAATCATAAGAGAAAAAAGATTTCAACCAATTTCCTACATTTCCAGAACATAG
                       L  N  M  G  I  I  R  E  K  R  F  Q  P  I  S  Y  I  S  R  T  *
Cow           TTGCGG  CTTAACATGGGGATCACAAGAGAAAAATGA
                       L  N  M  G  I  T  R  E  K  *
Dog           GAACAG  CTTAATATGGGAATCACAAGAGAAAAATGA
                       L  N  M  G  I  T  R  E  K  *
Shrew         TTACAG  CTTCACGTGGGAAGCACAAGGGAAAAGAATTTCTACCAATTTCCCACATTTCCAGAACATAGTGGCCTGTAG
                       L  H  V  G  S  T  R  E  K  N  F  Y  Q  F  P  T  F  P  E  H  S  G  L  *
Elephant      TTAGGG  CTTAACATGGACTCACAAGAGAAACACCATTTCAACCAATGTCCCACATTTCCAGACCACAGAGGCAGCTAG
                       L  N  M  D  S  Q  E  K  H  H  F  N  Q  C  P  T  F  P  D  H  R  G  S  *
C
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Figure 3. Alignments of amelogenin exons 4 and 4b, exons 5 and 8, and exon 9. **A**) Alignment of *AMELX* exon 4 with the putative exon "4b" sequence found in 5 rodent genomes. In 4 species, exon "4b" is putatively functional: correct donor (left) and acceptor (right) intron splices and sequence either identical or close to that of exon 4. In the kangaroo rat, exon "4b" is not coding, as indicated by the mutation of the acceptor intron splice (gray background); note that this exon "4b" sequence shows more nucleotide substitutions than in, *e.g.*, mouse and rat sequences. Nucleotide differences between exon 4 and exon "4b" are underlined. Latin names of species are indicated in Appendix 1. (**B**) Alignment of *AMELX* exon 5 with exon 8 sequences found in 5 rodent genomes. Mouse and rat sequences are functional. The 3 other exons 8 are putatively functional: correct donor (left) and acceptor (right) intron splices, no deleterious mutations, and sequence close to that of mouse exon 8. Nucleotide differences between exon 5 and exon 8 are underlined. Latin names of species are indicated in Appendix 1. (**C**) Alignment of functional (*i.e.*, found in mouse and rat *AMELX* transcripts, in bold), putatively functional (but no cDNA data), or non-coding (*i.e.*, putative intron splice mutated, gray background) *AMELX* exon 9 (nucleotide and protein sequences) of species representative of various mammalian lineages. Several sequences are putatively functional: correct donor intron splice (shown on the left) and beginning of the sequence similar to that of mouse and rat exon 9. Note the remarkable conservation of the sequence from elephant to mice, and the differences between human *AMELX* and *AMELY* sequences. * = stop codon. Latin names of species are indicated in Appendix 1.

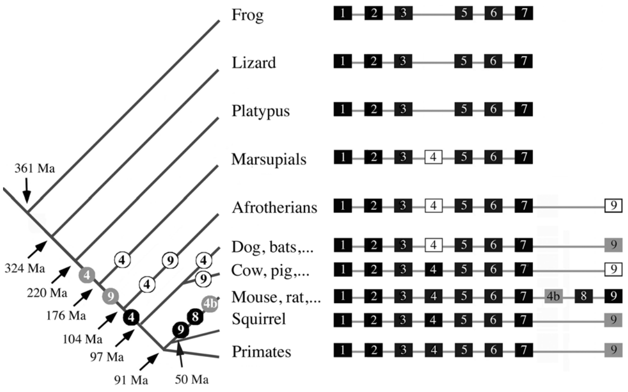

Figure 4. A summary of the story of AMELX small exons during mammalian evolution. **On the left:** putative location of the recruitment (numbered gray circles) then fixation (numbered black circles) or inactivation (numbered white circles) of *AMELX* exons 4, "4b", 8, and 9 during evolution. *AMELX* exon 4 was recruited first, in an ancestral therian, then exon 9 in an ancestral placental mammal. Exon "4b" and exon 8 were recruited in the lineage leading to murid rodents, around 50 million years (Ma) ago. Once created, these 4 small, mammalian-specific *AMELX* exons were conserved as either functional (fixed) or not (pseudogenic) in all subsequent mammalian lineages. **On the right**: For each lineage, *AMELX* organization is shown with functional exons as black blocks, pseudo-exons as white blocks, and putatively coding exons as gray blocks. Estimated times of divergence: tetrapods from Hedges (2009), amniotes from Shedlock and Edwards (2009), mammals from Madsen (2009), placental mammals from Murphy and Eizirik (2009), and rodents from Adkins *et al.* (2001) and Huchon *et al.* (2002).

Our results support the following scenario:

(1) Exon 4 appeared in an ancestral therian after a DNA region containing a similarly sized coding exon was duplicated. *AMELX* exon 5 is the most probable candidate, being close to exon 4 and having the same size. Additional exons are mostly recruited through the duplication of a DNA region within the same gene, as, *e.g.*, for the creation of exons "4b" and 8 in rodent *AMELX* (see below). A vast majority of such tandem duplications are likely to be involved in mutually exclusive alternative splicing events (Kondrashov and Koonin, 2001; Letunic *et al.*, 2002). Such an alternative splicing is known for exon 4.

(2) Once created from exon 5, the peptide encoded by exon 4 did not improve protein function (as redundant peptide), and it accumulated numerous substitutions until a functional copy was retained by natural selection in, *e.g.*, murids, primates, and artiodactyls; this process is expected after exon duplication, and could explain why sequence homology with exon 5 is no longer recognizable in all species possessing AMELX exon 4.

(3) Additional mutations occurred independently in therian lineages. Mutations affected the splice donor site, resulting in exon 4 inactivation in marsupials, afrotherians, xenarthrans, and some laurasiatherians. Given the short evolutionary period since exon 4 was inactivated, the sequence is still easily recognizable as pseudogenic.

(4) In some placental mammal lineages, exon 4 mutation resulted in a protein sequence somewhat useful for protein function (*e.g.*, useful when included in some particular transcripts), and these changes were fixed after positive selection, which occurred in an ancestor of primates, rodents, and artiodactyls, as revealed by the conserved exon 4 sequence in these species.

Eventually, exons "4b" and 8 were created in an ancestral rodent.

Coding or Not Coding Mammalian-specific Exons

In addition to providing information on the timing of the recruitment of these small exons during evolution, our analyses indicated whether these exons are putatively coding in representatives of the mammalian lineages. Indeed, current data concerning *AMELX* transcript sequences are available in a limited species only.

Exon 4

Exon 4 was identified in the first published human, mouse, rat, cow, and pig *AMELX* cDNAs (Gibson *et al.*, 1991; Salido *et al.*, 1992; Simmer, 1995). However, this exon is not encoded in the major AMELX isoform (known as A-4) (Veis, 2003). In rodents, the isoform containing the region encoded by exon 4 (called A+4) could have a different function compared with A-4, as suggested by bead implantation in the exposed pulp of rat molars. A-4 induced closure of the root canal, formation of a reparative dentinal bridge, and diffuse mineralization in the mesial part of the pulp chamber. The reaction was weaker after A+4 implantation (Six *et al.*, 2004; Jegat *et al.*, 2007). The positive selection of 5 residues during mammalian evolution means that these residues were fixed (no longer subjected to substitution) during mammalian evolution, suggesting that they have acquired a function important for this region of the protein.

Exons "4b" and 8

As mentioned before, exons "4b" and 8 are homologous to exons 4 and 5, respectively (Bartlett *et al.*, 2006). However, exon "4b" has not been identified in the various *AMELX* transcripts identified thus far in murids.

In the mouse, by sequencing the genomic region separating exons 7 and 8, we answered "no" to the question of exon "4b" being a possible artifact generated during computer-aided assembly of this genomic region. But, how to explain the identical sequences of exons "4b" and 4, and the absence of exon "4b" in *AMELX* transcripts? If the duplication/translocation event had occurred recently, *i.e.*, in the murine lineage, the resulting exon "4b" could have been non-coding without accumulating mutations during such a short time. However, we found that exons "4b" and 8 were created earlier, after the squirrel lineage diverged, *i.e.,* approximately 50 Ma ago. This is sufficiently long for mutations accumulating at random; otherwise, sequence conservation means that it is subjected to functional constraints, *i.e.,* it is coding. This finding is additionally supported when one considers the few nucleotide substitutions observed in exon "4b" compared with that observed in exon 8...that it is coding; both sequences were duplicated at the same time, but only exon 8 was found in several transcripts. Also, in the kangaroo rat, numerous substitutions were observed, while exon "4b" was inactivated.

However, although these findings indicate that exon "4b" should be coding, at least in rodents, we failed to find *AMELX* transcripts containing exon "4b" in a cDNA library of murine tooth buds. Either such a transcript is stage-specific during enamel formation or it is to be found in other loci in the mouse tooth. To date, the presence of exon "4b" in rodent *AMELX* gDNA remains an enigma.

Exon 9

In rodents, *AMELX* exon 9 encodes 9 residues and a stop codon. It was believed that the translocation of the gDNA region containing exons 4 and 5 downstream of exon 7 has triggered the activation of a downstream sequence, resulting in the expression of exon 9 (Bartlett *et al*., 2006). Here, we show that the exon 9 sequence was recruited long before rodent differentiation, in a placental mammal ancestor, 176-104 Ma ago. As discussed for exon 4, a gDNA region containing a coding exon was duplicated/translocated downstream of exon 7, mutations were accumulated at random, and fixation or inactivation occurred depending on whether the sequence was subjected to functional constraints.

However, why was exon 9 not found in mammalian *AMELX* transcripts other than in murids? The high similarity of exon 9 sequence in various mammals indicates that it should be coding, but our attempts to find transcripts including exon 9 in human tooth germs were unsuccessful. It is possible that such transcripts are stage-specific or expressed in other loci, or that human tooth enamel no longer requires the encoded peptide. Our cDNAs were prepared from human fetal tissue younger than 23 wks, when the tooth enamel was still in pre-secretory and early secretory stages. It is possible that exon 9 may not express at a detectable level at these stages. To date, the presence of an exon 9 sequence in non-murine *AMELX* gDNA also remains an enigma.

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