Amylase Gene Structures in Primates: Retroposon Insertions and Promoter Evolution

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Amylase transcription in the human salivary gland results from the evolutionary juxtaposition of two inserted elements, a γ-actin pseudogene and an endogenous retrovirus, to create an unusual salivary-specific promoter. We utilized these structures as molecular tags to characterize the amylase genes in extant primates by polymerase chain reaction amplification of promoter fragments from genomic DNA. Six distinct amylase promoter structures were identified, which allowed us to infer the structures of common ancestors and trace the evolution of the modern human amylase promoters. Our data show that integration of the pseudogene and retrovirus were evolutionarily recent events. The γ-actin pseudogene integrated after the divergence of the New World monkeys from the primate ancestral tree, and the retrovirus integrated later, after the divergence of the Old World monkeys. The New World monkey amylase promoter represents the mammalian amylase precursor structure before integration of the two retroposons. Two distinct amylase genes were identified in the Old World monkeys, one with a complete γ-actin pseudogene insert and another novel structure with a truncation of the γ-actin sequences. We demonstrated abundant amylase expression in the saliva of an Old World monkey, indicating that the endogenous retrovirus is not required for amylase transcription in the primate salivary gland.

Introduction

The human α-amylase gene family provides an excellent model system with which to investigate the evolutionary synthesis of a tissue-specific promoter. Molecular cloning revealed that distinct gene copies code for pancreatic and salivary amylase (Nishide et al. 1986). The complete gene family, clustered within a 250-kb region on human chromosome 1, contains five active genes, including two pancreatic genes (AMY2A and AMY2B) and three salivary genes (AMY1A, AMY1B, and AMY1C) (Gumucio et al. 1988; Samuelson et al. 1988; Groot et al. 1989). The pancreatic and salivary amylase promoters are highly related, with similar intron/exon boundaries and 98% nucleotide sequence identity over their coding regions (Horii et al. 1987). However, the salivary amylase genes contain an additional nontranslated exon at their 5' ends, and the pancreatic and salivary amylase promoters are unrelated. The gene structures are consistent with the theory that all five copies arose during evolution from a single ancestral gene through a series of duplications, with subsequent divergence of the promoter regions leading to differences in tissue-specific expression (Samuelson et al. 1990).

A striking feature of the human amylase genes is their close association with retroposon inserts. Three distinct promoter structures resulted from the independent insertion of two transposable elements, a processed γ-actin pseudogene and an endogenous retrovirus (Emi et al. 1988; Samuelson et al. 1988, 1990) (fig. 1). Each human amylase gene is associated with a γ-actin pseudogene, situated 0.2 kb upstream of the first coding exon (exon a). This insert has typical features of a processed pseudogene and is 91% identical to the human γ-actin cDNA sequence (Samuelson et al. 1990). The AMY2B gene is associated with a complete γ-actin pseudogene, approximately 2 kb in length; the other four amylase genes are associated with truncated pseudogene copies, missing the 5' two-thirds of the γ-actin sequence. The common pseudogene integration site in all five human amylase genes provides strong evidence that they arose by duplication of a single precursor gene following integration of the γ-actin.

The second retroposon identified in the human amylase gene cluster is an endogenous retrovirus (Emi et al. 1988; Samuelson et al. 1988). Four copies of the retrovirus are found within the cluster, interrupting the γ-actin pseudogene upstream of the three AMY1 genes and the AMY2A gene (fig. 1, filled boxes). The three salivary-specific AMY1 genes are associated with complete retroviral genomes, located 0.23 kb upstream from the transcriptional start site and in the opposite orientation to amylase. Remarkably, AMY1 transcription initiates within the γ-actin pseudogene, and the salivary amylase promoters are composites of pseudogene and retroviral sequences. The pancreatic AMY2A gene has retroviral sequences in the form of a solo long terminal repeat (LTR). However, transcription of this gene in the pancreas does not initiate within the pseudogene, but at a site downstream, in a position analogous to the start site of the other pancreatic gene, AMY2B (Samuelson et al. 1988). The AMY2B gene is not associated with a retroviral insert.

The close proximity of the γ-actin and retroviral inserts to the amylase promoters is unusual and suggests that integration of these retroposons may have influenced amylase expression. The importance of these elements for the regulation of salivary amylase transcription has been demonstrated by Ting et al. (1992) using gene transfer studies in transgenic mice. This study showed that a 1.0-kb AMY1C promoter fragment containing sequences derived
from the retroviral and γ-actin inserts was sufficient to
direct the expression of a reporter gene to the parotid sal-
vary gland. Furthermore, a 0.7-kb fragment derived solely
from the retrovirus was observed to direct salivary-specific
transcription when coupled to a heterologous promoter and
reporter gene. Thus, the provirus apparently contains sal-
vary-specific enhancer sequences. This result suggested
that the insertion of the retroviral and γ-actin transposons
upstream of an amylase gene during primate evolution re-
sulted in the formation of a new promoter active in the
salivary gland.

In this study we have investigated the evolutionary
synthesis of the human salivary amylase promoter
through the analysis of amylase gene structures in extant
primates. The unique nature of the retroposon inserts
allowed the use of γ-actin and retroviral sequences to
selectively amplify different amylase promoters from
primate genomic DNA by polymerase chain reaction
(PCR). Characterization of amylase promoters from rep-
resentatives of three major primate families, apes, Old
World monkeys, and New World monkeys, revealed the
evolutionary timing of the retroposon insertions. Our re-
results demonstrate that both the γ-actin and retroviral in-
sertions occurred after the divergence of the New World
monkeys from the primate ancestral tree, since the
amylase gene of these primates was not associated with
retroposons. All other primates studied contained the
amylase-associated γ-actin pseudogene, whereas only
the hominids, including apes and humans, contained
both the γ-actin pseudogene and the endogenous retro-
virus. A model for the evolution of the human salivary
amylase promoter is proposed.

Materials and Methods
Primate DNAs

Human DNA was purified from white blood cells
as described by Bell, Karam, and Rutter (1981). Non-
human primate DNA samples were generously provid-
ed by Drs. Jerry Slightom (UpJohn Co.), Kathy Neiswanger
(University of Pittsburgh), Richard Tashian (Uni-
versity of Michigan), and Ira Sampiao and Morris Good-
man (Wayne State University). The ape samples includ-
ed chimpanzee (Pan troglodytes), gorilla (Gorilla gorilla),
orangutan (Pongo pygmaeus), Mueller's gibbon
(Hylobates muelleri), and siamang (Hylobates syngap-
tylus). The Old World monkey samples included Japa-
nese macaque (Macaca fascicularis), rhesus macaque (Mac-
caca mulatta), cynomolgus macaque (Macaca fascicu-
laris), agile mangabey (Cercocebus galeritus), moustach-
ed guenon (Cercopithecus cephus), western black and
white colobus (Colobus polykomos), drill (Papio leuc-
ophaeus), and gelada baboon (Theropithecus gelada).
The New World monkey samples included common
squirrel monkey (Saimiri sciureus), long-haired spider
monkey (Ateles belzebuth), and saki (Pithecia irrorata).
Genomic DNA samples were quantitated with a TKO
100 fluorimeter (Hoefer), and integrity was assessed by
agarose gel electrophoresis and by PCR amplification
of an ε-globin control fragment, as described in Bailey,
Slightom, and Goodman (1992) (data not shown).
greater than 1.5 kb, samples were subjected to 35 cycles of 94°C for 25 set, 55°C for 30 set, and 72°C for 1.5

Table 2

Table 2
Nucleotide Sequences of Primers Used to Amplify/Sequence Primate Amylase Fragments

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Nucleotide Sequence (5' to 3')</th>
<th>Amylase Gene Region#</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a2a</td>
<td>GGTGTTTCCTCTTTTGGCATTTGCTAGGG</td>
<td>AMY2A (S; -1349 to -1322)</td>
<td>Samuelson et al. 1990</td>
</tr>
<tr>
<td>a2b ...</td>
<td>GGTACATCGTAGGTTGCTAGGG</td>
<td>AMY2B (S; -2.2 kb)</td>
<td>Samuelson et al. 1990</td>
</tr>
<tr>
<td>act ...</td>
<td>TCAGGTTGCAAAGCTCCACAGATTGGACC</td>
<td>AMY1 (S; +54 to +83)</td>
<td>Samuelson et al. 1988</td>
</tr>
<tr>
<td>aec ...</td>
<td>CIGTTACTGGTAGAAGCATAAGGCCCAGC</td>
<td>AMY1 (S; -277 to -250)</td>
<td>Samuelson et al. 1990</td>
</tr>
<tr>
<td>env ...</td>
<td>GCCTATCCAATCTATGGCAGAG</td>
<td>AMY1 (A; -85.8)</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>exa2 ...</td>
<td>TACTCGAGCCGCAGAACCACATG</td>
<td>AMY2 (A; +67 to +44)</td>
<td>Gumucio et al. 1988</td>
</tr>
<tr>
<td>ggg ...</td>
<td>GGTGAGGTGAGGAAATGGTAAAGACTGGTC</td>
<td>AMY1 (S; -991 to -962)</td>
<td>Ting et al. 1992</td>
</tr>
<tr>
<td>mac ...</td>
<td>CAAATCCAGAACCAGCTGCCCTGACAC</td>
<td>AMY2A (A; -536 to -563)</td>
<td>Samuelson et al. 1988</td>
</tr>
<tr>
<td>5ga ...</td>
<td>GAAGCTTGACGAGATGCTGAATATGGC</td>
<td>AMY2B (S; -914 to -884)</td>
<td>Samuelson et al. 1990</td>
</tr>
</tbody>
</table>

Note.—See figure 1 for a graphic illustration of the positions of the PCR primers in the human amylase promoters.

aAMY1 indicates the three salivary amylase genes (AMY1A, AMY1B, or AMY1C); AMY2 indicates the two pancreatic amylase genes (AMY2A or AMY2B). S = sense strand sequence; A = antisense strand sequence.

Table 2
Summary of the Amylase Gene Fragments PCR Amplified from Primate Genomes

<table>
<thead>
<tr>
<th>Fragment#</th>
<th>Primers</th>
<th>Primates#</th>
<th>Contents#</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMY2A-1.0</td>
<td>a2a/3ga</td>
<td>H, A, OW</td>
<td>amylose, γ-actin, LTR</td>
</tr>
<tr>
<td>AMY1-1.6.</td>
<td>gag/env</td>
<td>H, A</td>
<td>amylose, γ-actin, LTR</td>
</tr>
<tr>
<td>AMY1-0.7.</td>
<td>a2a/env</td>
<td>H, A</td>
<td>γ-actin, LTR</td>
</tr>
<tr>
<td>AMY2A-1.4</td>
<td>a2a/env</td>
<td>H, A</td>
<td>amylose, γ-actin, LTR</td>
</tr>
<tr>
<td>NWM-0.3</td>
<td>a2b/env</td>
<td>NWM</td>
<td>amylose</td>
</tr>
<tr>
<td>OWM-1.0</td>
<td>a2a/3ga</td>
<td>OW, OW</td>
<td>amylose, γ-actin</td>
</tr>
</tbody>
</table>

# See Materials and Methods for an explanation of fragment nomenclature.
# Fragments were amplified from a minimum of three species within each group. H = human, A = apes, OW = Old World monkeys, NWM = New World monkeys.
# Fragment contents were described by Southern hybridization with AMY (amylose), ACT (γ-actin), and LTR probes.

Amplification of Amylase Gene Fragments

Oligonucleotide primers were designed from human amylase sequences (fig. 1, table 1). PCR reactions were carried out in 50 μL containing 100–500 ng of genomic DNA; 10 mM Tris·HCl (pH 8.3); 50 mM KCl, 4 mM MgCl2; 0.01% (w/v) bovine serum albumin; 0.2 mM dATP, dCTP, dGTP, and dTTP (Pharmacia); and 0.2 μM of each primer and Taq polymerase. The a2a/exa2 amplification mix contained 0.5 mM MgCl2. Two different thermocycler programs were used. For fragments greater than 1.5 kb, samples were subjected to 35 cycles of 94°C for 25 sec, 55°C for 30 sec, and 72°C for 1.5 min, with a final extension step at 72°C for 5 min. For fragments less than 1.5 kb, samples were subjected to 35 cycles of 94°C for 20 sec, 55°C for 20 sec, and 72°C for 20 sec, with a final extension step at 72°C for 5 min. After amplification, the PCR products were subjected to agarose gel electrophoresis before capillary purification. Amplified strongly were diluted prior to agarose gel electrophoresis.

Southern Hybridization

Approximately 1 ng of each PCR product was resolved by agarose gel electrophoresis before capillary transfer to Zetaprobe membrane (BioRad). Membranes were hybridized with 32P-labeled DNA probes specific for amylase (AMY), γ-actin (ACT), or retroviral LTR sequences (fig. 1) at 60°C in hybridization solution (0.5 M NaPO4, 1 mM EDTA, 7% sodium dodecyl sulfate) containing denatured salmon sperm DNA (100 μg/mL). Linear DNA fragments used for probes were isolated from a plasmid subclone of cosmID G21 that contains the AMY1 gene (cosmid G21 is described in Gumucio et al. [1988]). Restriction fragments were purified after electrophoresis in low-melt agarose using the freeze fracture technique described by Qian and Wilkinson (1991). The AMY probe was a 0.45-kb EcoRI-NdeI I fragment that included exon α and approximately 200 bp of adjacent intronic sequence. The ACT probe was a 0.5-kb NdeI-HpaI fragment that included sequences from the 3' end of the γ-actin pseudogene. The LTR probe was a 0.24-kb Sac I-Bgl I fragment that included sequences from the 5' LTR of the primate virus. Probes were 32P-labeled using the random primer method of Feinberg and Vogelstein (1983). After hybridization overnight, membranes were washed at 60°C; the final wash solution contained 1× SSC (0.15 M NaCl and 0.15 M sodium citrate) and 0.1% sodium dodecyl sulfate. Genomic Southern analysis of human and cynomolgus macaque DNAs (10 μg) was performed as described in Samuelson, Isakov, and Lacourse (1995) using the AMY probe.

Amylase Activity in Saliva

Amylase enzyme activity was detected by acrylamide gel electrophoresis and starch-iodine staining using a modification of the procedure of Bloor and Meisler (1980). Saliva or serum samples were diluted in phosphate buffer (22 mM KH2PO4, 22 mM Na2HPO4, 7 mM NaCl, pH 6.9) to a total volume of 25 μL and amylase isozymes were resolved on a 7.5% acrylamide gel by electrophoresis overnight in buffer containing 12.5 mM Tris and 49 mM glycine (pH 8.1). Gels were subsequently incubated 30 min at 37°C in a solution of 2.5% starch (Sigma S-2630) in phosphate buffer (w/v), rinsed in water and stained with iodine solution (6 mM potassium iodide, 0.8 mM iodine). Amylase enzyme activity was observed as lightly staining areas on the gel.

Sequence Analysis

PCR products were isolated from low-melt agarose gels, purified by centrifugation through Millipore 30,000
NMWL Ultrafree-MC cellulose columns, and sequenced in the DNA Sequencing Core at the University of Michigan with an Applied Biosystems model 373A sequencer. Opposing primers were used to sequence both strands of each fragment.

Fragment Nomenclature

Eight distinct PCR-generated fragments were identified in this study (table 2). Fragments were named according to their size and the corresponding human amylace gene that exhibits this structure. For example, AMY1-1.6 refers to a 1.6-kb fragment amplified from an AMY1-like gene; AMY-0.6 refers to a 0.6-kb fragment common to all of the human amylase genes. NWM-0.3 refers to a PCR fragment specific to the New World monkeys and OWM-1.0 refers to a fragment specific to the Old World monkeys.

Results

Characterization of Amylase Promoter Structures in Nonhuman Primates

Amylase promoter fragments were amplified from the genomic DNAs of apes, Old World monkeys, and New World monkeys to test whether the retroposon insertions identified in the human amylase promoters were present in the nonhuman primates. Fifteen primate species were analyzed, including at least three representatives of each primate group (see Materials and Methods for a list of primate species). The PCR primers for this study were based on human sequence and were directed against different structural elements, including amylace coding sequences (exa2), γ-actin pseudogene sequences (act, 5ga), retroviral sequences (gag, env), and regions upstream of the two retroposon inserts (a2a, a2b) (fig. 1, table 1). The exa2 primer anchored all but one of the amplified fragments within the amylase coding region. The sequence of this region is identical in all five human amylase genes (Gumucio et al. 1988). The eight amplified fragments identified in this study are summarized in table 2 and described in detail below.

Identification of Primate Amylase Genes Associated with γ-Actin Pseudogenes

To estimate the timing of the γ-actin pseudogene insertion, we tested various New World monkeys, Old World monkeys, and apes for the unique juxtaposition of γ-actin and amylase sequences observed in the human genes. Primers complementary to amylase exonic
sequence (exa2) and γ-actin sequence (act) were used to amplify a 0.6-kb fragment (AMY-0.6) encompassing a region stretching from the γ-actin pseudogene to the first coding exon (exon a). The AMY-0.6 fragment is common to all five human amylase genes (fig. 1). The act/exa2 primer pair was demonstrated to amplify this fragment from human genomic DNA (fig. 2, lane 1) as well as from a recombinant plasmid clone containing the 5' end of the human AMY1C gene (lane 17). Analysis of fifteen different primate species demonstrated amplification of the AMY-0.6 fragment from apes (five species) and Old World monkeys (seven species), but not from New World monkeys (three species) (fig. 2). The structure of the amplified fragment was verified by Southern analysis, which demonstrated hybridization to the AMY and ACT probes (fig. 2), but not to the LTR probe (not shown). This result confirmed that this fragment included both amylase and γ-actin sequences. We did not detect any hybridization to the three New World monkey samples, demonstrating that even with the heightened sensitivity of Southern hybridization there was no evidence for amplification of the AMY-0.6 fragment from the genomes of these primates.

An AMY2B-like Gene is Present in Apes and Old World Monkeys

We next utilized the a2b primer to determine if a complete γ-actin pseudogene insert occurs upstream of the primate amylase genes. The a2b/exa2 primer pair amplifies a 2.2-kb fragment from genomes that contain an AMY2B-like gene. A product would not be expected from the AMY1 and AMY2A genes because they do not contain a complete γ-actin pseudogene insert or sequences complementary to primer a2b (fig. 1). When the primate DNAs were amplified with the a2b/exa2 primer pair, the AMY2B-2.2 fragment was observed in humans, five ape species, and six Old World monkey species (fig. 3). Southern hybridization with AMY and ACT probes confirmed that the fragment contained γ-actin and amylase sequences. The AMY2B-2.2 fragment was not amplified from the genomes of the New World monkeys; a 0.3-kb product was observed in its place. This length difference suggested that the 1.9-kb γ-actin pseudogene was missing from the New World monkey promoter. This was confirmed by Southern hybridization (fig. 3) and nucleotide sequence analysis (discussed below).
The amplification of the AMY-0.6 and AMY2B-2.2 fragments from the apes and Old World monkeys demonstrated that the γ-actin pseudogene insertion took place in a common ancestor. Furthermore, the absence of these fragments from the New World monkey samples suggested that the γ-actin pseudogene integrated sometime after the divergence of the New World monkeys. This interpretation was further supported using a third primer pair consisting of another γ-actin-specific primer (5ga) paired with exa2 to amplify a 1.0-kb fragment from genomes containing an AMY2B-like gene. As observed with the previous two primer pairs, the 5ga/ exa2 primer pair amplified a fragment (AMY2B-1.0) from the human, ape, and Old World monkey DNAs, but not from the New World monkey species (table 2; data not shown). Thus, we have consistently failed to amplify fragments indicative of an amylase-associated γ-actin pseudogene from the New World monkeys. This was not due to the inability of the common exa2 primer to align productively with the New World monkey am-

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**FIG. 4.**—The New World monkey amylase promoter does not contain a γ-actin pseudogene. (A) Structural comparison of the NWM-0.3 fragment amplified from the squirrel monkey with the AMY2B-2.2 fragment. (B) Comparison of the nucleotide sequence of the junction regions flanking the γ-actin insert with the squirrel monkey promoter. The uninterrupted squirrel monkey sequence is aligned with the 5' and 3' junctions of the γ-actin pseudogene insert (boxed) associated with AMY2B. (C) Nucleotide sequence alignment of the squirrel monkey amylase promoter with the human AMYZB and mouse Amy2.2 promoters. Nucleotide sequence identities (.) and deleted nucleotides (−) are indicated. The positions of the TATA box (double underlined) and transcription start site of the AMY2B gene in human pancreas (arrowhead) are indicated. Nucleotide numbering for the squirrel monkey gene is based on the human gene numbering. AMY2B sequence is from Gumucio et al. (1988) and Samuelson et al. (1990), Amy2.2 sequence is from Osborn et al. (1987).
ylase gene, since exa2 was observed to amplify the NWM-0.3 fragment when paired with the a2b primer (fig. 3, lanes 13–15).

The Structure and Sequence of the New World Monkey Amylase Promoter

We determined the nucleotide sequence of the squirrel monkey NWM-0.3 fragment to verify the structure predicted from its size and hybridization characteristics (fig. 3). As predicted, this promoter did not contain the γ-actin pseudogene (fig. 4). Comparison of the amylase promoter sequences downstream of the pseudogene insert showed that the squirrel monkey amylase promoter is 93% identical to the human AMY2B promoter and 73% identical to the mouse pancreatic amylase promoter (fig. 4C). The mouse pancreatic amylase promoter also does not contain retropon inserted (Osborn et al. 1987). The structural similarity between the New World monkey and murine pancreatic amylase promoters suggests that an amylase gene without a γ-actin pseudogene represents the mammalian precursor structure.

Identification of Primate Amylase Genes Associated with Retroviral Inserts

To test for the presence of the amylase-associated retrovirus, the gag/exa2 primers were used to amplify a 1.6-kb fragment indicative of an AMY1-like structure. The AMY1-1.6 fragment was amplified from human genomic DNA and the structure was verified by Southern hybridization with AMY, ACT, and LTR probes (fig. 5). When the nonhuman primate species were tested, the AMY1-1.6 fragment was amplified from three ape species (chimpanzee, gorilla, and orangutan), but was not amplified from Old World monkeys (seven species) or New World monkeys (three species). The AMY1-1.6 fragment includes proviral 5′ LTR and gag sequences. To test for the presence of the 3′ end of the retrovirus we utilized a primer from the env region paired with a primer upstream of the inserts (a2a). The a2a/env primer pair amplified a 0.7-kb fragment from human and ape genomic DNA, but not from the Old World and New World monkeys (table 2; data not shown). This result is consistent with the previous pattern of amplification observed with the gag/exa2 primer pair, and indicates that the apes, but not the more distantly related monkey species, contain an AMY1-like gene that is associated with a complete endogenous retrovirus.

An AMY2A-like Gene is Present in the Apes

To check for the presence of an AMY2A-like gene, we paired the a2a flanking primer with the amylase exonic primer exa2 to amplify a 1.4-kb fragment that includes the γ-actin pseudogene and retroviral LTR characteristic of this gene structure. The AMY2A-1.4 fragment was amplified from human DNA (fig. 6, lane 1) and its structure was verified by hybridization with AMY, ACT, and LTR probes (data not shown). The pattern of amplification of the AMY2A-1.4 fragment followed the results of amplification of the other fragments containing retroviral sequences: the fragment was detected in the genomes of apes (chimpanzee, gorilla, and orangutan), but was not observed in Old World monkeys (five species) or New World monkeys (three species) (fig. 6). Thus, the results of amplification with the gag/exa2, a2a/env, and a2a/exa2 primer pairs were consistent with the interpretation that insertion of the endogenous retrovirus into the primate amylase cluster occurred sometime after the divergence of the New and Old World monkeys and before the divergence of the apes.

The Structure and Nucleotide Sequence of a Novel Amylase Promoter in Old World Monkeys

In place of the expected AMY2A-1.4 fragment, the a2a/exa2 primer pair amplified a 1.0-kb fragment from the five Old World monkey species (fig. 6, lanes 5–9). Southern blot analysis demonstrated that this OWM-1.0 fragment hybridized to the AMY and ACT probes, but not to the LTR probe (data not shown). Based on its size and hybridization characteristics, this fragment was hypothesized to represent a novel amylase gene structure containing a truncated γ-actin pseudogene and no retroviral insert. The nucleotide sequence of the Japanese...
macaque OWM-1.0 fragment was determined with the amplification primers a2a and exa2, and two internal primers, mact and act2 (fig. 7). The sequence data confirmed that this gene resembles an AMY2A-like gene lacking the retroviral insert, since the macaque sequence could be readily aligned with the human AMY2A gene sequence upstream and downstream of the LTR. This result also supports our previous conclusion that the endogenous retroviral insert is only associated with the amylase genes of the apes and humans.

The macaque amylase promoter sequence (−196 to +4) is compared to three other primate amylase promoters in figure 7C. This comparison includes the human pancreatic genes AMY2A and AMY2B, as well as the squirrel monkey amylase sequence determined in this study. The macaque sequence is 90% and 88% identical to the human and squirrel monkey sequences, respectively. There is especially strong sequence conservation surrounding the putative TATA box; in the region encompassing −57 to +1 the genes are ≥96% identical. There are several additional segments showing complete sequence identity extending 10 nt or longer, indicating that they may have functional significance. Evolutionarily conserved sequences of similar length in the primate globin promoters have been demonstrated to be binding sites for transcription factors (Gumucio et al. 1992, 1994).

Abundant Salivary Amylase Expression in the Macaque

Two distinct amylase promoters were PCR amplified from the genomes of the Old World monkeys with the AMY2B-2.2 and OWM-1.0 fragments (table 2). To determine if these two promoters represented the total complement of amylase genes in the genomes of these primates, we performed Southern analysis with EcoR1-digested cynomolgus macaque DNA. When hybridized with the AMY probe, we detected two hybridizing fragments, 3.2 kb and 7.3 kb in size, corresponding to two amylase genes (fig. 8). The 3.2-kb band comigrated with the human AMY2B gene fragment, suggesting that the 7.3-kb band originated from the novel gene associated with a truncated α-actin pseudogene. These data confirmed that our PCR approach identified the total complement of Old World monkey amylase promoters.

We next tested whether the macaque amylase genes were expressed in the salivary gland by measuring amylase activity in saliva. Amylase enzyme activity was detected by starch-iodine staining after polyacrylamide gel electrophoresis (fig. 9). Comparable activity was observed in the monkey (lane 3) and human (lane 4) saliva samples, which were collected and processed identically. Furthermore, the
monkey sample exhibited two distinct bands of activity, suggesting that they may be products of the two different amylase genes. Since the retrovirus is not associated with the Old World monkey amylase promoters, we conclude from this result that the retrovirus is not required for salivary amylase expression.

**Discussion**

In this study we have characterized the amylase promoters in extant primates to infer the structures of amylase gene precursors and predict the pathway of evolution of the human salivary amylase promoter. We test-
ed for the presence of the γ-actin and endogenous retroviral inserts characteristic of the human amylase promoters by PCR amplification of genomic DNA fragments from apes, Old World monkeys, and New World monkeys. Our results demonstrated that both the γ-actin and retroviral elements inserted into the amylase gene cluster during primate evolution. The results are summarized in figure 10, which illustrates the evolutionary relationships between the primate families, and the amylase gene structures identified in each group. Our data indicate that the γ-actin insertion occurred after the divergence of the New World monkeys and before the divergence of the Old World monkeys from the human-ape lineage. This was demonstrated by amplification of the γ-actin-containing amylase promoter fragments AMY-0.6, AMY2B-2.2, and AMY2B-1.0 from humans, apes, and Old World monkeys, but not from New World monkeys (table 2). These results suggest that the pseudogene insertion took place 23–45 million years ago (MYA) (Goodman 1986). This time frame is in general agreement with a 40 MYA estimate based on molecular clock analysis of sequence divergence of the γ-actin pseudogene (Samuelson et al. 1990). Our data also indicate that the endogenous retrovirus inserted into the amylase-associated γ-actin pseudogene sometime later in the human-ape lineage, after the divergence of both the New and Old World monkeys. This was demonstrat-

![Fig. 8.—Macaques contain two distinct amylase genes. Genomic Southern analysis of human (lane 2) and cynomolgus macaque (lane 3) DNAs digested with EcoRI and hybridized with the AMY probe. The sizes in kb of the hybridizing amylase fragments identified from the cloned human genes are indicated at the right. The sizes of the BRL 1 kb molecular weight standard (lane 1) are indicated on the left.](https://academic.oup.com/mbe/article-abstract/13/6/767/1023448/8)

![Fig. 9.—High level of amylase enzyme activity in cynomolgus macaque saliva. Samples were subjected to polyacrylamide gel electrophoresis and stained for amylase enzyme activity as described in Materials and Methods. The positions of the human pancreatic (AMY2) and salivary (AMY1) amylase isozymes observed in human serum (lane 1) are indicated on the left. The positions of the two bands of amylase activity observed in macaque saliva (lane 3) are indicated on the right. Lanes: 1, human serum (10 μL); 2, fresh human saliva (0.05 μL); 3, macaque saliva (0.005 μL); 4, human saliva (0.005 μL) collected and processed along with the macaque sample.](https://academic.oup.com/mbe/article-abstract/13/6/767/1023448/9)
The final step in our model is the insertion of the endogenous retrovirus into the truncated \(\gamma\)-actin pseudogene to create the \(AMY1\) gene structure observed in the human. This proposed pathway predicts a switch in tissue-specific expression, since the five human gene copies that formed from a single precursor exhibit divergent expression in the pancreas and salivary gland (Samuelson et al. 1988).

Gene expression studies in transgenic mice have demonstrated that salivary-specific transcriptional control elements are located within the amylase-associated provirus, as evidenced by the parotid-specific expression of a transgene containing a 0.7-kb proviral fragment (Ting et al. 1992). This result suggested that salivary specificity is encoded by the retrovirus, and that amylase expression in the salivary gland was determined upon retroviral integration in the primate lineages. However, our demonstration of high levels of amylase activity in the saliva of the macaque contradicts this idea, and instead indicates that salivary amylase expression predated the retroviral insertion (fig. 8). Two additional Old World monkey species, the rhesus and mangabey, have also been reported to contain high levels of amylase in their saliva (McGeachin and Adin 1982), indicating that salivary amylase expression is a general property of this primate group. Our analysis showed that neither Old World monkey amylase promoter contained the amylase-associated retrovirus (figs. 8 and 10).

A summary of the amylase promoter structures detected in this study is diagrammed with the evolutionary relationships of the primate families containing these structures. Promoter structures are labeled with the corresponding human amylase gene; novel promoter structures are unlabeled. The structures detected in the apes (chimpanzee, gorilla, and orangutan) were similar to the previously described human genes. The proposed timing of insertion of the \(\gamma\)-actin and retroviral retroposons are indicated on the phylogenetic tree on the right. Salivary amylase expression in each primate group is indicated (+ or −) (Junqueira, Toledo, and Doine 1973; McGeachin and Adin 1982). With the exception of the human genes, the identification of salivary amylase promoters is unknown. The minimal \(AMY1\) gene region (0.7 kb) that confers salivary-specific expression in transgenic mice is indicated by the bar (from Ting et al. 1992). Open boxes, amylase exons; shaded boxes, \(\gamma\)-actin pseudogene; filled boxes, endogenous retrovirus.

Fig. 10.—A summary of the amylase promoter structures detected in this study is diagrammed with the evolutionary relationships of the primate families containing these structures. Promoter structures are labeled with the corresponding human amylase gene; novel promoter structures are unlabeled. The structures detected in the apes (chimpanzee, gorilla, and orangutan) were similar to the previously described human genes. The proposed timing of insertion of the \(\gamma\)-actin and retroviral retroposons are indicated on the phylogenetic tree on the right. Salivary amylase expression in each primate group is indicated (+ or −) (Junqueira, Toledo, and Doine 1973; McGeachin and Adin 1982). With the exception of the human genes, the identification of salivary amylase promoters is unknown. The minimal \(AMY1\) gene region (0.7 kb) that confers salivary-specific expression in transgenic mice is indicated by the bar (from Ting et al. 1992). Open boxes, amylase exons; shaded boxes, \(\gamma\)-actin pseudogene; filled boxes, endogenous retrovirus. A summary of the amylase promoter structures detected in this study is diagrammed with the evolutionary relationships of the primate families containing these structures. Promoter structures are labeled with the corresponding human amylase gene; novel promoter structures are unlabeled. The structures detected in the apes (chimpanzee, gorilla, and orangutan) were similar to the previously described human genes. The proposed timing of insertion of the \(\gamma\)-actin and retroviral retroposons are indicated on the phylogenetic tree on the right. Salivary amylase expression in each primate group is indicated (+ or −) (Junqueira, Toledo, and Doine 1973; McGeachin and Adin 1982). With the exception of the human genes, the identification of salivary amylase promoters is unknown. The minimal \(AMY1\) gene region (0.7 kb) that confers salivary-specific expression in transgenic mice is indicated by the bar (from Ting et al. 1992). Open boxes, amylase exons; shaded boxes, \(\gamma\)-actin pseudogene; filled boxes, endogenous retrovirus.
ected in modern retroposons may not relate to the mechanism of initial change in gene expression.

All vertebrates express amylase in the pancreas, while expression in the salivary gland is limited to some but not all species of primates, rodents, lagomorphs, and chiropterans (Junqueira et al. 1973). There are two possible models to explain this pattern (Meisler and Gumucio, 1986). One model predicts that both salivary and pancreatic amylase existed in the mammalian ancestor with extinction of salivary amylase in several lineages during evolution. Alternatively, the mammalian ancestor might have contained only pancreatic amylase, with salivary expression being acquired independently in certain lineages. Support for independent acquisition of salivary amylase in the rodents and primates is provided by the observation that the human and mouse salivary amylase promoters are unrelated in structure or DNA sequence (Gumucio et al. 1988). The mouse promoter is associated with a nontranslated exon located 7 kb upstream of exon a (Young, Hagendtichle, and Schibler 1981). In contrast, the human salivary amylase promoter is associated with a nontranslated exon located 0.5 kb upstream of exon a. Furthermore, the human but not the mouse salivary amylase promoter is the product of the insertion of two transposable elements. Despite the divergent nature of the human and mouse salivary amylase promot ers, human salivary amylase transgenes were expressed in the mouse with the proper tissue specificity (Ting et al. 1992). The nature of the salivary-specific transcription factors regulating amylase expression in the salivary gland has not yet been characterized.

Independent acquisition of salivary amylase in rodents and primates would predict that salivary amylase expression can be favored by natural selection. Although the nature of an evolutionary advantage afforded by salivary amylase activity is unclear, it has been hypothesized that the agreeable taste of sugars produced by amylase enzymatic activity in the mouth could help animals recognize nutritious food sources (Ting et al. 1992). Any increase in digestive efficiency or food recognition could be favored by selection over time.

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