Am. J. Hum. Genet. 50:303–316, 1992

The Human Enamel Protein Gene Amelogenin Is Expressed from Both the X and the Y Chromosomes

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Summary

Amelogenins, a family of extracellular matrix proteins of the dental enamel, are transiently but abundantly expressed by ameloblasts during tooth development. Amelogenins seem to regulate the formation of crystallites during the secretory stage of enamel development, while they are specifically degraded during tooth-bud maturation. In this paper we report the characterization of the AMGX and AMGY genes on the short arms of the human X and Y chromosomes which encode the amelogenins. Our studies on the expression of the amelogenin genes in male developing tooth buds showed that both the AMGX and AMGY genes are transcriptionally active and encode potentially functional proteins. We have isolated genomic and cDNA clones from both the AMGX and AMGY loci and have studied the sequence organization of these two genes. Reverse transcriptase (RT)PCR amplification of the 5′ portion of the amelogenin transcripts revealed several alternatively spliced products. The splicing pattern observed in the Y-derived mRNA varies from that of the X-derived mRNA. The promoter regions from both genes and the predicted amelogenin protein sequences are presented. This information will be useful for studying the molecular basis of X-linked amelogenesis imperfecta, for understanding the evolution and regulation of gene expression on the mammalian sex chromosomes, and for investigating the role of amelogenin genes during tooth development.

Introduction

Amelogenins and enamelins are the two major classes of proteins in the extracellular matrix of developing dental enamel (Termine et al. 1980). They are secreted by the ectoderm-derived ameloblasts in tooth buds during early stages of odontogenesis. The profile of the enamel matrix proteins changes during tooth formation. Early enamel contains predominantly the proline-rich amelogenins which are selectively metabolized during enamel maturation, leaving their degradation products and the acidic enamelins in the mature enamel (Fincham 1980; Robinson et al. 1983). Amelogenins are thought to regulate the formation of enamel crystallites, probably by providing the hydrophobic environment necessary for the initiation and growth of calcium hydroxyapatite crystals (Deutsch 1989). The nature and function of enamelins are not completely clear (Robinson et al. 1989); recent reports claim that the major forms of enamelins are in fact albumin and other serum proteins (Strawich and Glimcher 1990).

Amelogenin cDNA clones have been isolated from both bovine and murine cells (Snead et al. 1985; Shimokawa et al. 1987*a*). Using the mouse cDNA as a probe, Lau et al. (1989) have mapped the mouse amelogenin gene to the X chromosome. In humans, sequences homologous to the mouse amelogenin cDNA have been found on the short arm of the X chromosome in the p22.1-p22.3 region (the AMGX locus) and in the pericentric region of the Y chromosome (the AMGY locus). Using linkage analysis, La-

Received July 25, 1991; revision received October 17, 1991.

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0002-9297/92/5002-0007$02.00

gerström et al. (1990) have mapped X-linked amelogenesis imperfecta (AI), a genetic disorder affecting the formation of enamel, to Xp22, suggesting that defects in the amelogenin structural gene may be the cause of X-linked AI (AIH1). In fact, a deletion in the amelogenin gene has been recently found in a family with AIH1 (Lagerström et al. 1991). It is not known whether the AMGY locus encodes a functional protein. We therefore proceeded to study the expression of the AMGY locus in human male developing tooth buds.

Because fetal tooth buds are difficult to obtain for isolation of mRNA and because the DNA sequence of the human amelogenin gene is unknown, we first used a mouse amelogenin cDNA probe to isolate human genomic sequences from the AMGX and AMGY loci. We then used the exon sequences shared by the sex chromosomes, to design primers for reverse transcriptase for (RT)–PCR amplification of the amelogenin transcripts in male fetal tooth buds. We were able to differentiate the PCR products by X- or Y-specific oligonucleotide probes or by specific restriction enzymes. Eventually we isolated and characterized overlapping clones containing full-length cDNAs from both chromosomes. Our results indicate that the AMGY locus on the Y chromosome encodes a functional protein, although its level of expression is only 10% of that of the AMGX locus on the X chromosome.

Material and Methods

General Procedures

The screening of a human genomic library, the preparation of phage and plasmid DNA, and the gel electrophoresis and Southern blotting of restriction fragments were all carried out according to standard procedures (Sambrook et al. 1989). DNA sequences were determined using the dideoxy chain-termination method using double-stranded DNA as template (Chen and Seeburg 1985).

Isolation and Analysis of mRNA from Human Fetal Tooth Buds

Incisive tooth buds from six human fetuses ranging from 19 to 24 wk of gestation were collected during a postmortem examination. Samples were immediately frozen in liquid nitrogen, and RNA was extracted by the guanidium isothiocyanate method (Sambrook et al. 1989). One microgram of each RNA sample was electrophoresed in formaldehyde containing agarose and blotted onto a nylon membrane. Two samples, corresponding to a male fetus and a female fetus, showed best preservation of RNA and gave a strong autoradiographic band after hybridization with a mouse amelogenin cDNA probe. The male sample was used to generate human amelogenin cDNA.

Cloning of the Human Amelogenin cDNA

The 5′ and the 3′ portions of the human amelogenin transcripts were cloned separately using the method developed by Frohman et al. (1988). To clone the 3′ portion of the human amelogenin cDNA, 5 μg of total RNA from fetal tooth buds was reverse transcribed using 0.5 μg of the dT_{17}-adapter (5′-GACTCGAGTCGACATCGAT_{17}) as primer. The reaction product was diluted 1:250 with TE, and the amelogenin cDNA was PCR amplified using primers Pram6 from the gene and the adapter (5′-GACTCGAGTCGACATCGA-3′), at 1 pmol/μl each. After an initial denaturation step at 95°C for 5 min, annealing at 50°C for 2 min, and extension at 72°C for 40 min, 30 step-grade cycles were performed in a Perkin Elmer–Cetus thermal cycler set at 94°C for 40 s, at 57°C for 2 min, and at 72°C for 3 min, with a final extension at 72°C for 15 min. The PCR product was electrophoresed in 1.5% agarose and was blotted and hybridized with a mouse amelogenin cDNA probe.

The PCR product was either blunted with the Klenow fragment of DNA polymerase and ligated to pUC-19 digested with *Sma*I or digested with *Pst*I and *Sal*I and ligated to pUC-19 digested with *Pst*I and *Sal*I. Ligation products were used to transform JM83 *Escherichia coli* made competent by the calcium chloride method (Sambrook et al. 1989). Recombinant colonies were replated and screened with X- and Y-specific oligonucleotide probes according to a method described elsewhere (Wallace and Miyada 1987; Buluwela et al. 1989). Several X- and Y-specific colonies were amplified, and their plasmid DNA was purified by the cesium chloride method. DNAs from X- and Y-specific colonies were sequenced with Sequenase (U.S. Biochemical).

To clone the 5′ half of the human amelogenin cDNA, 5 μg of total RNA were reverse transcribed by priming with an internal primer (Pram7) according to a method described elsewhere (Frohman et al. 1988). Excess primers were removed with a Centricon 100 cartridge, and the product of reverse transcription was tailed with terminal deoxynucleotide transferase and dATP. Samples (1 pmol/μl) of each internal primer

(Pram5) and adapter were used to amplify the tailed DNA by PCR, as described. The PCR product was electrophoresed in 1.5% agarose and was blotted onto nylon and hybridized with a mouse amelogenin cDNA probe. After the PCR product was blunted with Klenow DNA polymerase, it was ligated to pUC19 digested with *Sma*I. The product of ligation was used to transform competent *E. coli* strain JM83 cells. Recombinants were replated and screened with a mouse amelogenin cDNA probe. Representative positive colonies were sequenced as described above.

Quantitation of the Amelogenin Y-specific Transcripts

To investigate the presence of Y-specific transcripts, the PCR product of the 3′ half of the amelogenin cDNA obtained above was digested with two restriction enzymes, *Eco*RII and *Hae*III, that, compared with the X, have an additional site in the Y genomic sequence. Digests were electrophoresed in 8% acrylamide and electroblotted on a nylon membrane. This membrane was hybridized with an X-specific oligonucleotide probe (Pram21) at 57°C according to a method described elsewhere (Wallace and Miyada 1987). After the X-ray film was exposed for ~1 h, the probe was stripped, and the membrane was reprobed with a Y-specific oligonucleotide probe (Pram10) at 61°C.

To determine the relative abundance of X and Y transcripts, the PCR amplification was performed in the presence of 1 μl each of ^{32}P-dCTP and ^{32}P-TTP. The PCR product was digested with either *Eco*RII or *Hae*III and electrophoresed in 8% acrylamide. The X- and Y-specific fragments were cut and counted. The linearity of the counting was established by repeating the analysis with the PCR product spiked with one-tenth the amount of ^{32}P-dCTP and ^{32}P-TTP.

Flourescence In Situ Hybridization

Metaphase spreads were prepared from a PHA-stimulated, methotrexate-synchronized peripheral blood culture according to a method described by Yunis (1976). Slides had been stored in nitrogen bags at −20°C for 6 mo. The DNA probe was biotinylated by nick translation using a BioNick™ labeling system (BRL cat. 8247SA). Hybridization conditions were according to published procedures (Pinkel et al. 1986), with some modifications. In brief, slides were treated with pepsin (20 μg/ml in 0.1 N HCl) for 10 min at 37°C before denaturation in 70% formamide, 2 × SSC for 5–10 min at 70°C. Probes were hybridized at 4 ng/μl in a solution containing 50% formamide, 2 × SSC, 10% dextran sulfate, pH 7.0, and 1 μg sonicated total human placental DNA (~300 bp)/μl and were denatured at 70°C for 5 min and kept in ice until applied to each denatured slide. Slides were then incubated at 37°C for ~17 h. Slides were washed and stained according to published procedures (Trask et al. 1991), with some modifications. In brief, slides were washed in 50% formamide, 2 × SSC thrice and 0.1 × SSC once at 45°C for 15 min each. Slides were rinsed with 4 × SSC for 5 min and preblocked with 4 × SSC, 1% BSA for 5 min. Avidin-FITC (Vector; cat. A-2011) was applied at 5 μg/ml in 4 × SSC, 1% BSA for 30–45 min at room temperature. Slides were sequentially washed in 4 × SSC, 4 × SSC, 0.1% Tween 20, 4 × SSC, PN buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1% NP-40, pH 8) for 10 min each at room temperature. Slides were preblocked with PNM buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1% NP-40, pH 8, 5% nonfat dry milk) for 5 min and were exposed to 5 μg biotinylated anti-avidin antibody (Vector, cat. BA-0300)/ml in PNM buffer for 30–45 min at room temperature. Slides were washed in PN buffer thrice for 5 min each at room temperature, were preblocked with PNM buffer for 5 min, and were applied with 5 μg Avidin-FITC/ml in PNM buffer for 30–45 min at room temperature. Slides were then washed in PN buffer thrice at room temperature for 10 min each and were mounted with DAPI (0.4 μM) or PI (0.2 μg/ml) in antifade solution.

A Nikon microscope (Optiphot-2) equipped with UFX-DX camera and four-filter adaptor (Technology, Newark, NJ) was used for photography. Filter combinations are EX 330–380 (excitation), DM400 (dichroic), and BA 450 (barrier) for DAPI and are EX450–490, DM 510, and BA 520 for FITC. The cells were exposed under these two filter combinations sequentially without film advance, in order to locate signals over chromosomes.

Results

Isolation of Human Amelogenin Genomic Sequences

A human genomic library (Clontech HL1006) was screened with a probe pGEM17-2 containing 669 bases of the 3′ portion of the mouse amelogenin cDNA. Three positive clones (Ham 1, Ham 2, and Ham 3) were isolated. On the basis of size of the *Eco*RI fragments which hybridized to pGEM17-2, Ham 1 and Ham 3 are derived from the X chromosome, while Ham 2 is derived from the Y chromosome. Restriction

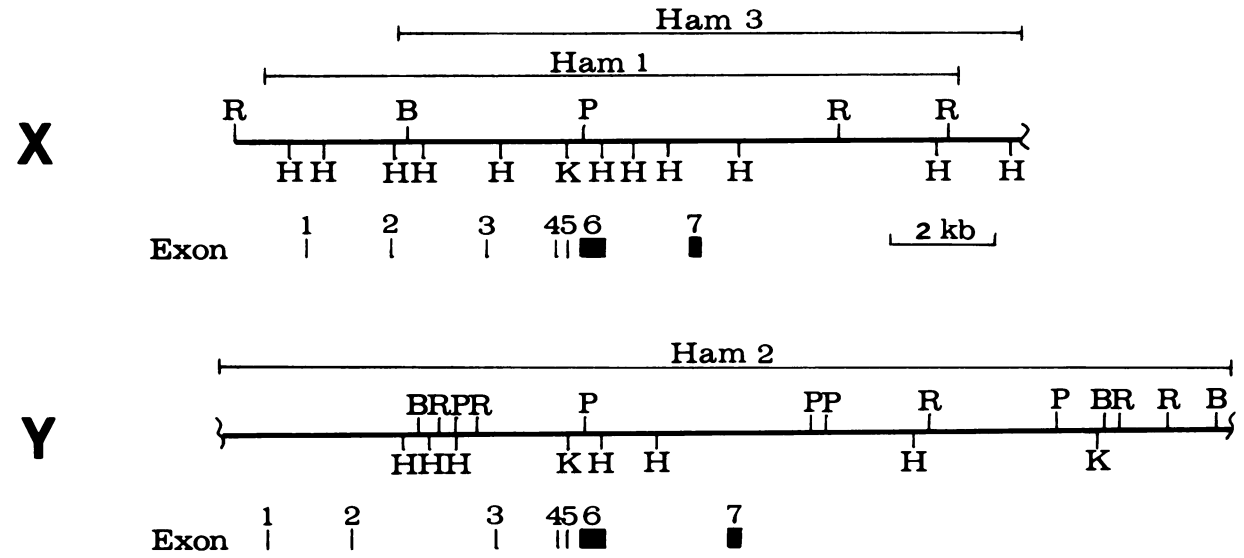


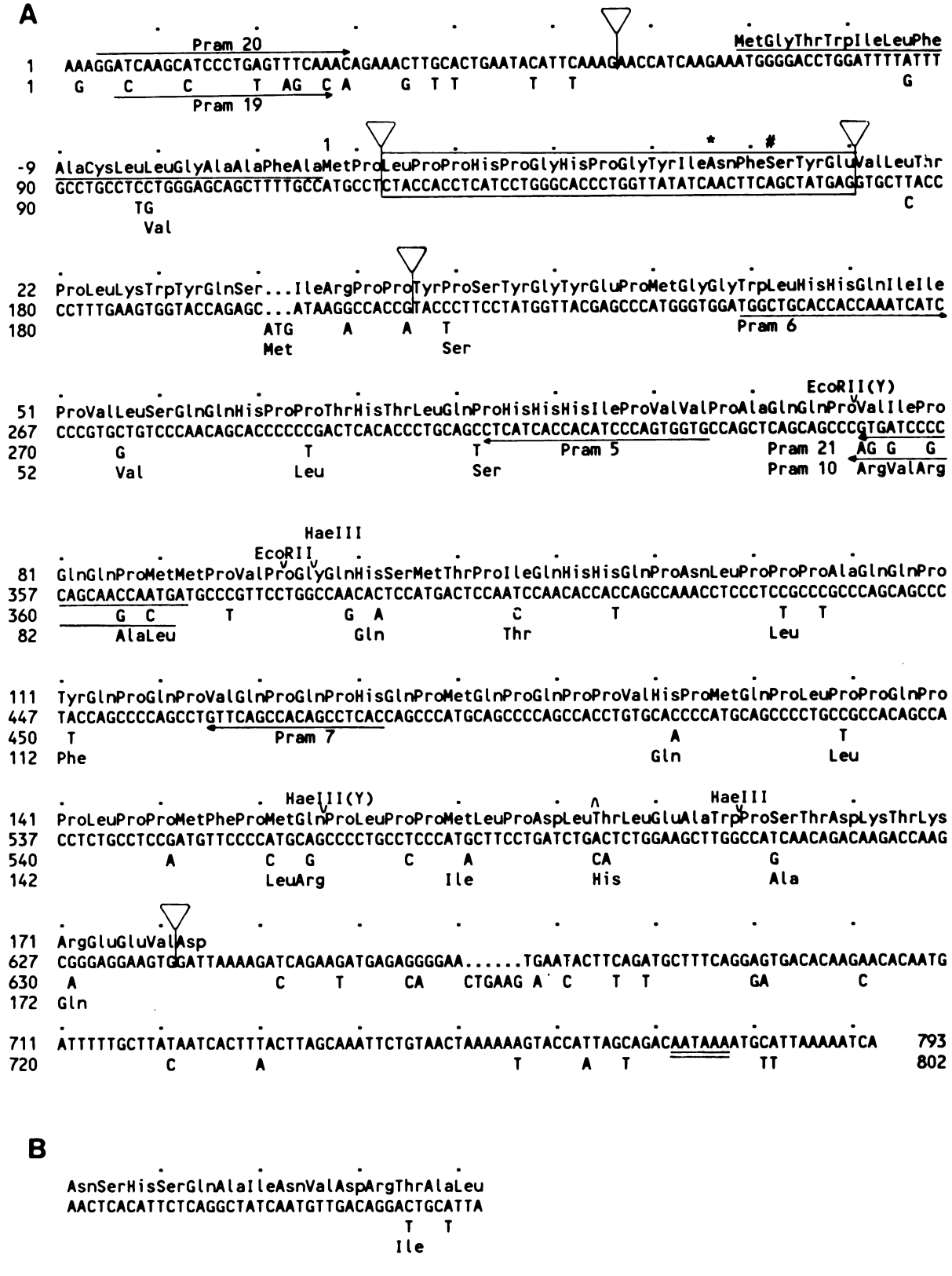
Figure 1 Structural organization of human amelogenin genes on X and Y chromosomes. Recombinant phages containing human inserts from the AMGX and AMGY loci are shown above the respective restriction maps, and the locations of the exons are shown below. B = *Bam*HI; H = *Hin*dIII; K = *Kpn*I; P = *Pst*I; and R = *Eco*RI.

maps of these clones were constructed (fig. 1), and the regions hybridizing with pGEM17-2 were sequenced. Two exons, subsequently found to be exons 5 and 6, were identified on both the X and the Y clones. Primers were designed from sequences shared by both chromosomes, to be used in the RT-PCR amplification of the amelogenin transcripts. In addition, chromosome-specific probes were made from a region (nucleotides 347–370) where the X chromosome and the Y chromosome differ in 6 of 19 bases. Sequence analysis of exon 6 revealed that the Y exon should contain additional *Eco*RII and *Hae*III sites which are absent in the X exon. These sites were used to differentiate the X and Y RT-PCR products as described below. The locations of the PCR primers, the chromosome-specific probes, and the sites for *Eco*RII and *Hae*III are shown in figure 2.

Expression of the Human Amelogenin mRNA from both the X and the Y Chromosomes

Total cellular RNA was isolated from the incisive tooth buds of a male fetus at 24 wk gestation. Northern hybridization of the RNA with pGEM17-2 showed a major transcript of 0.9 kb (data not shown). The RNA was reverse transcribed into cDNA by using a primer containing oligo dT linked to an adapter sequence (Frohman et al. 1988). The 3′ end of the amelogenin cDNA was then amplified by PCR using both a primer containing the adapter sequence and an internal primer Pram6 containing sequence common to both the X and the Y chromosomes (fig. 2). Gel electrophoresis of the PCR products showed a main fragment of 550 bp which hybridized with pGEM17-2 (data not shown). Restriction enzymes *Eco*RII and

Figure 2 Nucleotide and deduced amino acid sequences of human amelogenin cDNAs from AMGX and AMGY loci. *A*, DNA and protein sequences of major species of amelogenin. The X sequences are shown with the Y sequences indicated below whereever they are different from the X sequences. The various PCR primers and chromosomal specific probes, as well as *Eco*RII and *Hae*III sites used to differentiate the X and Y transcripts, are indicated. The amino acid residues are numbered from the methionine residue found at the N terminus of the mature protein. The signal peptide is indicated by single solid underlining. The region (exon 3) that is deleted in 16% of the AMGX 5′ cDNA clones is enclosed by a box, and the polyadenylation signal is indicated by a double solid line. The triangles indicate the places where the introns interrupt the gene. The sites for N-linked glycosylation, serine phosphorylation, and threonine phosphorylation are indicated by an asterisk (*), a number symbol (#), and a circumflex (^), respectively. *B*, Nucleotide and deduced peptide sequences of exon 4. This exon was inserted after nucleotide 170 in the above sequence found in one of the AMGX 5′ cDNA clones.


A
Pram 20
MetGlyThrTrpIleLeuPhe
Pram 19
AlaCysLeuLeuGlyAlaAlaPheAlaMetProLeuProProHisProGlyHisProGlyTyrIleAsnPheSerTyrGluValLeuThr
ProLeuLysTrpTyrGlnSer...IleArgProProTyrProSerTyrGlyTyrGluProMetGlyGlyTrpLeuHisHisGlnIleIle
Pram 6
EcoRII(Y)
ProValLeuSerGlnGlnHisProProThrHisThrLeuGlnProHisHisHisIleProValValProAlaGlnGlnProValIlePro
Pram 5
Pram 21
Pram 10
ArgValArg
HaeIII
EcoRII
GlnGlnProMetMetProValProGlyGlnHisSerMetThrProIleGlnHisHisGlnProAsnLeuProProProAlaGlnGlnPro
AlaLeu
Gln
Thr
Leu
TyrGlnProGlnProValGlnProGlnProHisGlnProMetGlnProGlnProProValHisProMetGlnProLeuProProGlnPro
Pram 7
Phe
Gln
Leu
HaeIII(Y)
HaeIII
ProLeuProProMetPheProMetGlnProLeuProProMetLeuProAspLeuThrLeuGluAlaTrpProSerThrAspLysThrLys
LeuArg
Ile
His
Ala
ArgGluGluValAsp
Gln
ATTTTTGCTTATAATCACTTTACTTAGCAAATTCTGTAACTAAAAAAGTACCATTAGCAGACAATAAAATGCATTAAAAATCA
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B
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Ile

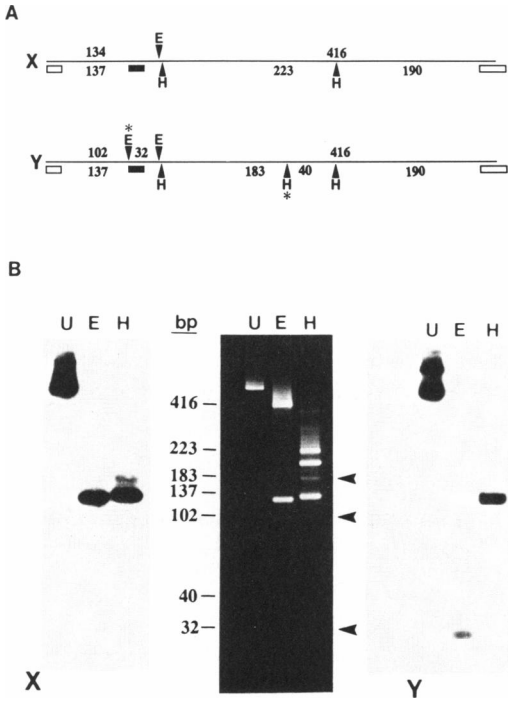

Figure 3 Expression of amelogenin transcripts from both X and Y chromosomes. *A*, Differentiation of AMGX and AMGY transcripts with restriction enzymes. The 3′ portion of the amelogenin transcripts in male tooth buds was RT-PCR amplified with primers (Pram6 and the adapter sequence) denoted by unblackened boxes. The locations of *Eco*RII (E) and *Hae*III (H) sites as well as the predicted fragment sizes (in bp) are shown. Sites that are unique to the Y transcripts are labeled with an asterisk (*). Blackened boxes indicate the locations of the chromosome-specific oligonucleotide probes used. *B*, Gel electrophoresis and Southern analyses of 3′ portion of amelogenin transcripts in male tooth buds. The central panel shows an ethidium bromide–stained 8% polyacrylamide gel of the RT-PCR products. U = undigested PCR products; E = *Eco*RII-digested products; and H = *Hae*III-digested products. The sizes (in bp) of the fragments are indicated on the left. Arrows on the right point to some of the Y-specific fragments. The gel was blotted with either an X-specific oligonucleotide probe Pram21 (*left panel*) or a Y-specific probe Pram10 (*right panel*). See the text for details.

*Hae*III were used to differentiate the X- and Y-derived PCR products, and the predicted fragments are indicated in figure 3*A*. The chromosomal origin of some of the fragments was confirmed by their hybridization with the X- or Y-specific oligonucleotides Pram21 and Pram10, respectively. As shown in figure 3*B*, *Eco*RII digestion of the PCR products gave a 416-bp common fragment, a 134-bp fragment hybridizing only with the X-specific probe, a faint 102-bp Y fragment, and a 32-bp fragment which hybridized only with the Y-specific probe. The presence of Y-specific transcripts was further confirmed by the *Hae*III digestion, which gave the predicted 183-bp Y-specific fragment. Our results indicate that the amelogenin sequence on the Y chromosome is transcribed, although at a level much lower than that of its X homologue. To quantitate the relative abundance of the X and Y amelogenin transcripts, ^{32}P-labeled PCR products were prepared, and the X- and Y-specific restriction fragments were excised from the gel and counted. As shown in table 1, the Y transcripts amount to ~10% of the total amelogenin transcripts, whether the comparison was made between the *Eco*RII fragments or between the *Hae*III fragments.

Table 1

Quantitation of X and Y Amelogenin Transcripts

DNA Fragment[a] (chromosomal origin)	cpm	cpm/bp	Y/(Y+X)
E134 (X)	21,706	161.9	7.6%
E102 (Y)	1,360	13.3	
H223 (X)	54,230	243.1	9.5%
H183 (Y)	4,700	25.6	
H137 (X+Y)	31,317	228.6	11.2%

[a] Restriction fragment of RT-PCR products of 3′ portion of amelogenin transcripts. The letter denotes the restriction enzyme (E = *Eco*RII; H = *Hae*III), and the number is the size in base pairs.

Cloning Human Amelogenin cDNAs

The presence of AMGY transcripts does not prove that the AMGY locus encodes a functional protein. We therefore proceeded to isolate full-length amelogenin cDNAs from the X and the Y chromosomes. The 5′ and 3′ halves of the human amelogenin cDNA were cloned separately using the method developed by Frohman et al. (1988). The RT-PCR products containing the 3′ half of the cDNA obtained above were cloned into pUC19. Recombinant clones were screened with the X- and Y-specific probes Pram21 and Pram10, respectively. Forty-three X clones and four Y clones were identified, consistent with the relative abundance of the X and Y transcripts as determined above. DNA from representative clones was isolated and sequenced.

To clone the 5′ half of human amelogenin cDNA, male tooth-bud RNA was reverse transcribed using primer Pram7 containing common sequences from the

3′ portion of the gene (fig. 2). The cDNA was tailed with oligo dA and was PCR amplified using an internal primer Pram5 and the oligo dT-adapter primer. The PCR products were cloned in pUC19, and transformants hybridizing with pGEM17-2 were identified. Thirty clones were analyzed by DNA sequencing, and 28 of them contained X-specific sequences while two contained ligated X and Y sequences. The majority of the X clones began from the same nucleotide at their 5′ ends, suggesting that they contain the cap site of the transcripts. Three different species of AMGX cDNA clones which are the results of alternative splicing have been isolated.

Since neither of the 5′ Y sequences obtained above extended to the 5′ end of the mRNA, the PCR amplification of the 5′ half of the amelogenin transcripts was repeated using a Y-specific primer Pram10 instead of the common primer Pram7. Twenty clones were isolated, and, of the nine clones sequenced, four contained Y sequences starting from the cap site.

Comparison of the Amelogenin cDNAs and Derived Proteins from the X and the Y Chromosomes

The nucleotide sequences of the major species of amelogenin cDNAs from both the AMGX and AMGY loci, along with their derived protein sequences, are shown in figure 2. Compared with the AMGY gene, the AMGX gene contains two small deletions; one is an ATG codon for methionine at nucleotide 201, and the other is a 6-bp deletion in the 3′ untranslated region. The full-length transcripts from the AMGX and AGMY loci are 793 bp and 802 bp in size, respectively, not including the poly A tail. The first in-frame ATG codon occurs at nucleotide 69 on both chromosomes, although the published protein sequences of mature human amelogenin start from methionine encoded by the ATG codon at nucleotide 117 (Fincham et al. 1990). Neither of these ATG codons is flanked by the translational start consensus sequence CANC (Kozak 1984; Cavener 1987). However, the peptide sequence following the first methionine is characteristic of a signal peptide, and it is highly homologous to the signal peptide predicted from the bovine amelogenin cDNA (fig. 4; Shimokawa et al. 1987*a*). In addition, a hydrophobic region, the hallmark of signal sequences (Gierasch 1989), is present upstream from the ATG codon at nucleotide 117. It is therefore very likely that amelogenins are synthesized as precursors with signal peptides of 16 residues which are cleaved off when the proteins are secreted into the extracellular matrix. The mature amelogenin from the X chromosome is predicted to be a protein of 19.8 kD with 175 amino acid residues, while the Y isoform is a 20-kD protein with 176 amino acids. The predicted molecular weights of amelogenins agree with the observed molecular weights of 25–30 kD, when the posttranslational glycosylation and phosphorylation of the protein are taken in account. The 3′ untranslated regions of the X and Y transcripts are 189 and 195 bp, respectively, and there is an AATAAA polyadenylation signal 15 bp upstream of the poly A tail. In addition, there is a diffuse GT-rich sequence which represents the second element of the mammalian poly A signal downstream from the 3′ end of the mRNA (Proudfoot 1991).

Three different species of AMGX 5′ cDNA clones have been identified among the 28 X clones characterized. Twenty clones contain the sequence shown in figure 2*A*. Five clones have a deletion of 48 bp from nucleotides 123–170 within the coding region, resulting in a "truncated" protein missing residues 3–18. One clone has an insertion of 42 bp after nucleotide 170, resulting in the addition of 14 amino acid residues shown in figure 2*B*. Only one species of AMGY cDNA was isolated initially. However, from the RT-PCR amplification of the 5′ portion of the amelogenin transcripts described below, it can be inferred that the AMGY transcripts are also alternatively spliced.

The amelogenin protein contains several structural domains: a signal peptide consisting of 16 residues, a highly conserved amino terminus domain, a core domain rich in Gln-Pro residues, and a hydrophilic C-terminus consisting of 12 residues which is rapidly removed after the protein is secreted into the extracellular matrix (Takaki et al. 1984). Although the overall sequence similarity between the AMGX and AMGY cDNAs is 91%, it varies significantly between the coding and noncoding regions, as shown in table 2. While the protein coding regions (exons 2–6) are highly conserved, with a similarity index between 93% and 100%, the 5′ and 3′ untranslated regions (exons 1 and 7) are much less conserved.

Figure 4 shows a comparison of the amelogenin protein sequences from human, mouse (Snead et al. 1985), cattle (Shimokawa et al. 1987*a*) and pig (Fukae and Shimizu 1983). The first 28 amino acids at the N terminus are identical. The methionine at residue 29 of the human Y amelogenin is conserved in other mammalian amelogenins but is absent from the predicted human X amelogenin, because of a 3-bp deletion in the human X amelogenin gene. Both a conserved consensus sequence (NFS) for N-linked glyco-

Table 2

Exon-Intron Organizations of Human Amelogenin Genes

No.	Exon Size (base)	Intron Size (kb)	Sequence of Exon-Intron Junctions			Similarity Between X and Y (exon/intron)
			5′ Boundary	Intron	3′ Boundary	
1	56	1.5	T(C/T)A AAG (56)	gtatgtgg ..... catttcag	AAC CAT	76.7/76.0
2	66	1.8/2.7	ATG CCT (122) Pro (2)	gtgagtaa..... t(g/a)taaaag	CTA CCA Leu (3)	95.4/83.4
3	48	1.3/1.1	TAT GAG (170) Glu (18)	gtaatttt ..... t(t/c)ttgtag	AAC TCA Asn	100/93.3
4	42	.091	GC(A/T) TTA Leu	gtgagtct ..... ctcttaag	GTG CT(T/C) Val (19)	95.2/91.1
5	42/45	.27	CCA CC(G/A) (212/215) Pro (32/33)	gtatgtag .....ctcaccag	TAC (C/T)CT Tyr (33/34)	95.1/94.0
6	426	1.7/2.2	GAA GTG (638/641) Val (174/175)	gtgagta(t/c) ..... tttttcag	GAT TAA Asp (175/176)	93.2/87.7
7	~160					88.4/. . .

[a] Nucleotide sequences of exons are in capital letters, while those of introns are in lowercase letters. The numbers following the nucleotides refer to the cDNA nucleotide positions where introns interrupt the amelogenin mRNA. The corresponding amino acids, with their positions in parentheses, are shown below the nucleotide sequences. When the nucleotides or positions are different for the X and Y genes, they are separated by a slash (i.e., X/Y). Sequence homology between the X and Y genes was studied using the BESTFIT program of UWGCG, and the similarity index between corresponding X and Y exons is separated from that between corresponding X and Y introns by a slash (i.e., exon/intron).

sylation at residue 14 and a conserved consensus sequence (SYE) for serine protein phosphorylation at residue 16 are present in all the amelogenins. An additional threonine phosphorylation site (TLE) is present in the predicted sequence of human X amelogenin. Within the central domain of the protein, the presence of 15 Gln-Pro repeats in the X amelogenin and of 13 Gln-Pro repeats in the Y amelogenin is noted. A 12-amino-acid hydrophilic domain that constitutes the carboxy terminus of the amelogenin is also conserved. This domain includes a dibasic motif (KR) at residue 171 in the predicted sequence of human X amelogenin, a motif which is absent from the human Y amelogenin.

Sequence Organization of the Human Amelogenin Genes

The human amelogenin cDNA clones isolated as described above were used to determine the exon/intron organization of the AMGX and AMGY genes. Both genes contain seven exons spanning >9 kb. The gene structures are depicted in figure 1, and characteristics of the exons and introns are listed in table 2. The structural organization of the AMGX and AMGY loci are very similar, with the introns interrupting the genes at the same locations. However, some of the introns are of different sizes. As mentioned above, three different species of cDNA clones from the X gene have been identified which are generated by alternative splicing. Exon 4 is present in only one of 28 AMGX cDNA clones studied. The majority of the cDNA clones contain the remaining six exons, while 16% of the cDNA clones lack exon 3. Although the only species of AMGY cDNA isolated lacks exon 4 sequences, a putative exon 4 with intact splice sites is present in the Y locus, and experiments described below show that exon 4 is present in some of the AMGY transcripts. It is noted that all the exons end with codon triplets, so that the alternative splicing does not change any amino acid residues.

Sequence similarities between the X and Y exons and introns are shown in table 2. When the introns are large, only ~300 bp flanking the exons were sequenced. There is no significant difference between the exons and introns in the X/Y comparison.

Figure 5 shows the 5′ promoter regions of the amelogenin genes. The transcription initiation site is indicated by an asterisk. There is a putative TATA box at position −25 and a reversed CCAAT box at −51 on both chromosomes. Such a reversed CCAAT box has been found at the 5′ promoter region of another extracellular matrix protein, the αI(IV) collagen (Killen et al. 1988). The X and Y genes share 80% similarity in the 5′ upstream regions.

Nakahori et al. (1991*a*) have recently isolated hu-

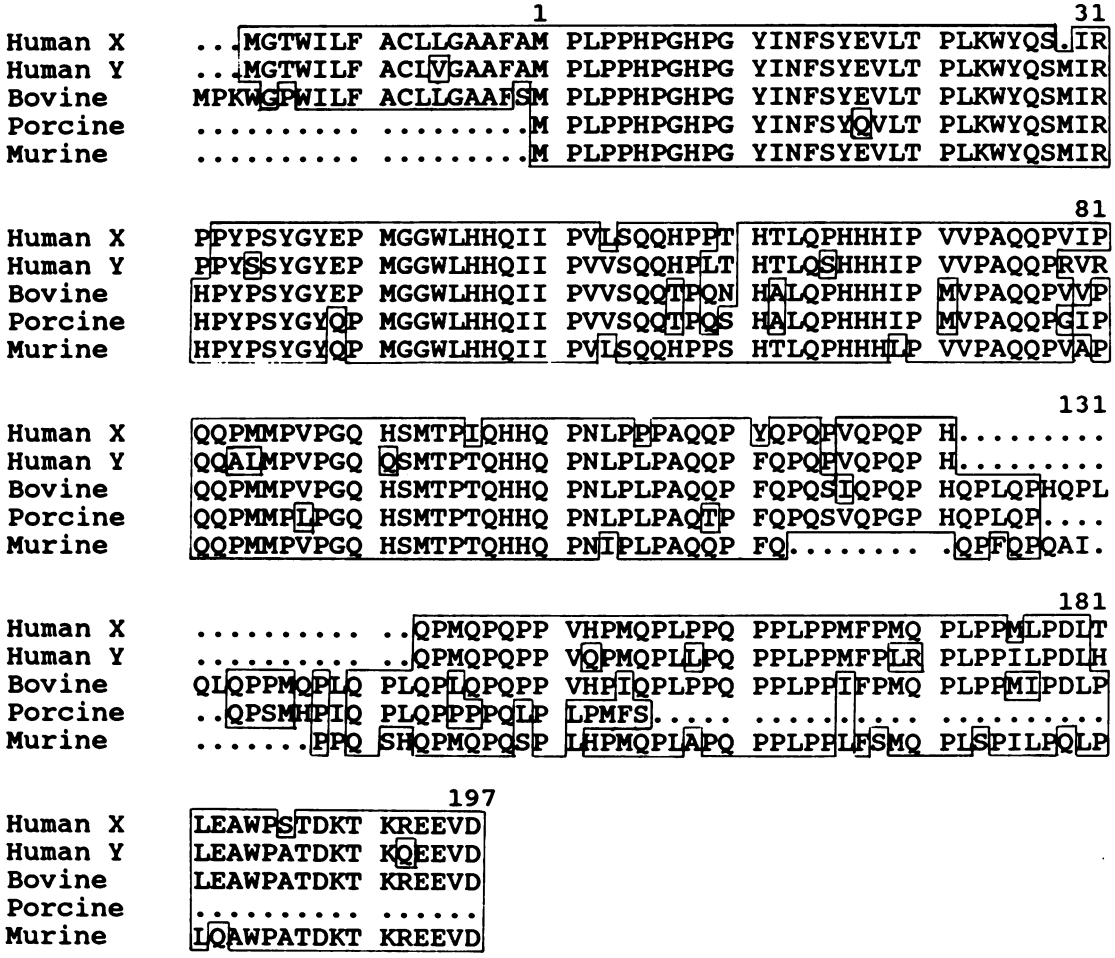

Figure 4 Comparison of protein sequences of amelogenins from human, cattle, pig, and mouse. The methionine residue found at the N-terminus of secreted amelogenins is labeled 1. No information is available on the signal peptide sequences of porcine and murine amelogenins. Sequences of the entire porcine amelogenin and of the N-terminal portion of the mouse amelogenin were determined solely by protein sequencing, while the rest were derived from cDNA sequences partially supplemented with protein sequencing. Sequences were aligned using the BESTFIT program from the UWGCG package, and identical residues are boxed.

man genomic clones containing amelogenin sequences and have identified exons 3, 5, and 6 in the 2.8 kb of DNA sequences they determined. Their sequences agree with ours, except that in their sequences 15 extra nucleotides are included as the 3′ end of exon 6.

The AMGX and AMGY Transcripts Are Differentially Spliced

The isolation of three species of AMGX 5′ cDNA clones suggests that the AMGX transcripts are alternatively spliced. Although only one species of AMGY 5′ cDNA was isolated initially, the close resemblance of the AMGY gene to the AMGX gene suggests that AMGY transcripts may also be alternatively spliced. We therefore studied the splicing of amelogenin transcripts in detail by using the technique of RT-PCR. The 3′ portion of the amelogenin genes does not seem to be involved in alternative splicing, since RT-PCR amplification of the 3′ portion of the amelogenin transcripts reveals only one product (fig. 3*B*). In addition, only one species of 3′ cDNA has been isolated. To study alternative splicing of the 5′ portion of the genes, we have designed two pairs of chromosome-specific primers, one pair of which contains sequences from exon 1 and one pair of which contains sequences from exon 6 (fig. 2). Primers Pram10-Pram19 amplify the 5′ portion of the AMGY transcripts, while primers

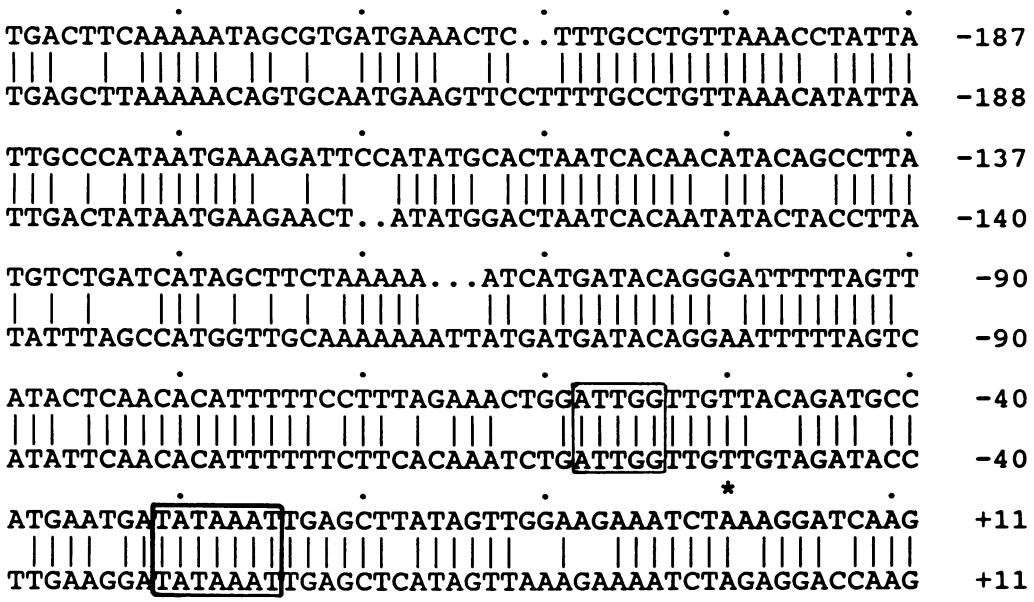

Figure 5 Sequence comparison of 5′ upstream regions of AMGX and AMGY genes. The transcription initiation site is indicated by an asterisk (*) Bars between the sequences indicate identical bases. A putative TATA box at position −25 and a reversed CCAAT box at −51, which are present in both the X and Y genes, are enclosed in boxes.

Pram20-Pram21 amplify that of the AMGX transcripts. The chromosome specificity of the primers was verified using cDNA clones pX_4 and pY_4 containing the AMGX and AMGY sequences, respectively. The X-specific primers amplified only pX_4 (but not pY_4) sequences, while the Y-specific primers amplified only pY_4 sequences (fig. 6). Total tooth-bud RNAs isolated from a male and a female fetus were reverse transcribed using Pram7 as primer and were PCR amplified using the X- or Y-specific primer pair. Gel electrophoresis analysis revealed several species of PCR products (fig. 6), and all of them hybridized with an AMGX 5′ cDNA clone (data not shown). The male and female RNAs gave the same PCR products when the X-specific primers were used. The major fragment (band 5) is of the same size as are the PCR products of pY_4 and pX_4, which lack exon 4. Band 3 corresponds to the PCR product of pX_3, which contains exon 4. Band 5 very likely represents transcripts missing exons 3 and 4, as we have found in five of the AMGX cDNA clones. The relative intensities of bands 3–5 appear to agree with the number of the various AMGX 5′ cDNA clones we isolated. Band 1 and band 2 are likely to be PCR artifacts due to heteroduplex formation (Nagamine et al. 1989), because further amplification of isolated bands 1 and 2 with the X-specific primers gave the same PCR products as did the starting tooth-bud mRNA. PCR amplification of the male mRNA by using the Y-specific primers produced a fragment (band 6) which was absent when X-specific primers were used. In addition, there is a faint band slightly larger than band 5. Cloning and sequencing of band 6 showed that it lacks exons 3–5. The faint band above band 5 therefore very likely represents transcripts missing exons 4 and 5. It is noted that exon 3 and exon 5 are 48 and 42 bp, respectively. Our studies using chromosome-specific primers to amplify the 5′ portion of the amelogenin transcripts therefore showed that the AMGX and AMGY transcripts are differentially spliced.

In situ Localization of the AMGY Locus

The amelogenin sequence on the human Y chromosome has been tentatively mapped to the proximal long arm in the Yq11 region, on the basis of the retention of the AMGY locus in a fibroblast cell line GM-1709 which was derived from a phenotypic female with an intact X chromosome and an isochromosome for the long arm of the Y chromosome (Lau et al. 1989). However, the aberrant Y chromosome in GM-1709 has been redefined recently as dicentric with a breakpoint in the p11.2 region (NIGMS Human

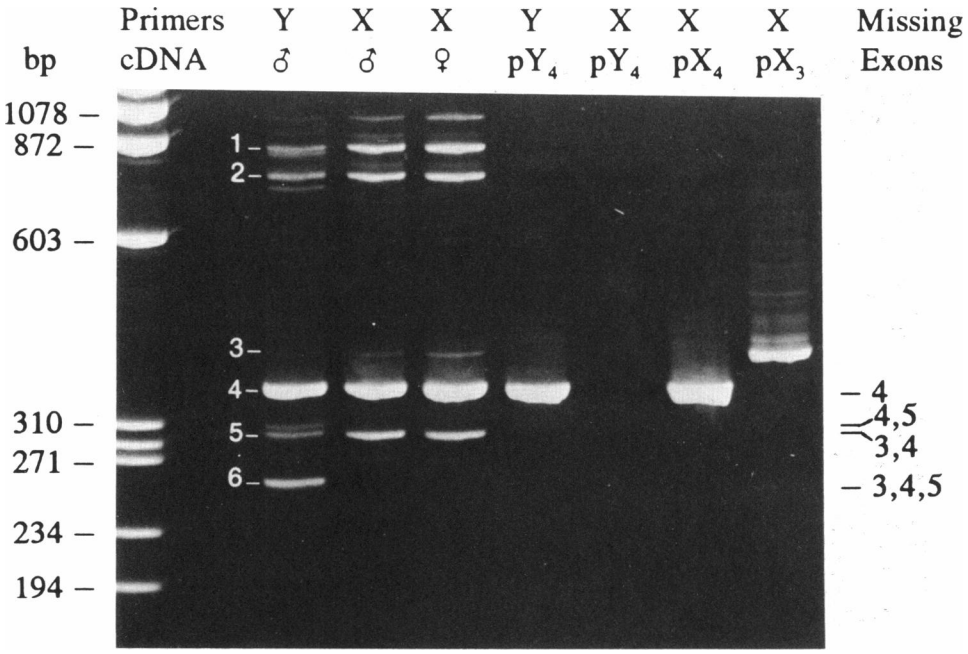

Figure 6 Alternative splicing of AMGX and AMGY transcripts. Male (♂) and female (♀) tooth-bud cDNAs, as well as amelogenin cDNA clones containing the Y (pY_4) or X (pX_4 and pX_3) sequences, were PCR amplified using either Y-specific primers (Y) or X-specific primers (X) containing sequences from exons 1 and 6 of the genes. The PCR products were separated on a 5% acrylamide gel and were stained with ethidium bromide. DNA length markers are shown on the far left, and exons missing in the various PCR fragments are indicated on the right. Band 6 is present only when the Y-specific primers are used, indicating that the AMGX and AMGY transcripts are differentially spliced. See the text for details.

Genetic Mutant Cell Repository 1990/1991 catalog of cell lines), thus raising the possibility that the AMGY locus may be on the short arm of the Y chromosome. We therefore mapped the AMGY locus by fluorescent in situ hybridization using the AMGY genomic clone Ham 2 as a probe. Twenty-one metaphase Y chromosomes with hybridization signals have been examined and the AMGY locus is clearly on the short arm of the Y chromosome in the p11.2 region (fig. 7).

Discussion

In the present study we have demonstrated that the AMGY locus on the Y chromosome is transcriptionally active in developing human tooth buds. By using a RT-PCR-based procedure (Frohman et al. 1988), we have cloned both AMGX and AMGY cDNAs, starting from minute amounts of human tooth-bud RNA. We estimated that in male tooth buds, the AMGX transcripts are 10 times more abundant than the AMGY ones and that both transcripts code for functional proteins. The ratio of AMGX to AMGY 3′ cDNA clones isolated was consistent with this estimate. Since we have studied only one male fetus at 24 wk gestation, we do not know whether this ratio stays the same during the development of the tooth or whether this ratio is consistent among different individuals. In the mouse, amelogenin expression has been shown to follow a spatially and temporally restricted pattern during development of the teeth (Snead et al. 1988), and thus the relative amounts of X- and Y-derived transcripts which we observed may reflect the single point in fetal life that we were able to sample. The difference in the transcription activities of the AMGX and AMGY loci very likely resides in their promoter regions, which share only 80% sequence similarity. Both promoters contain identical TATA and CCAAT boxes. However, other cis-acting elements which may control the transcriptional level of the amelogenin genes have not been identified. Studies on the amelogenin promoters may require a cell line derived from ameloblasts, which are the only cell type known to express amelogenin genes.

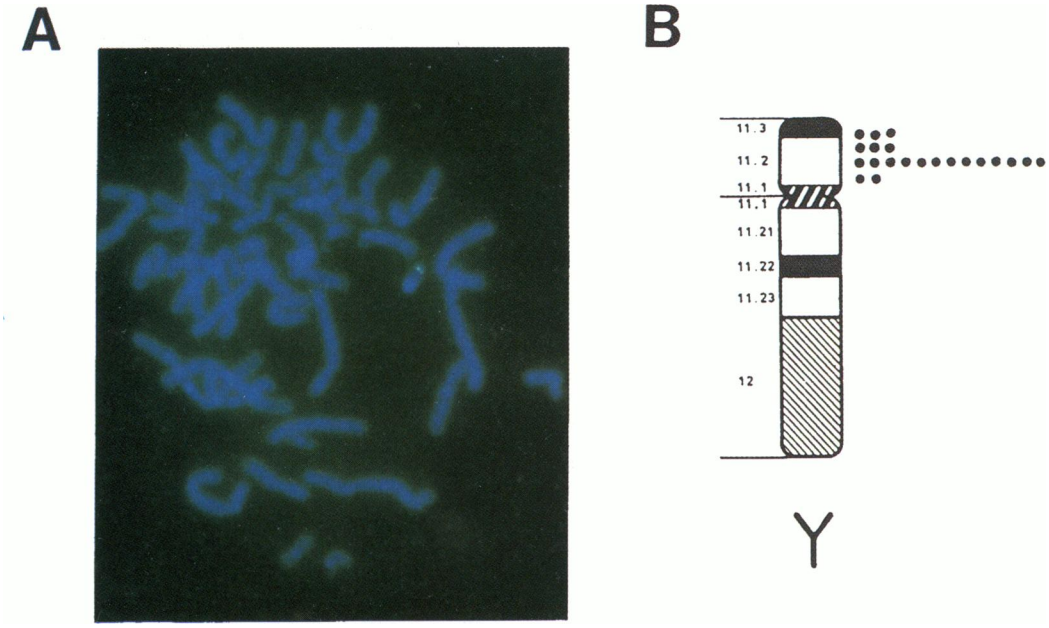

Figure 7 Mapping AMGY locus to Yp11.2 by fluorescent in situ hybridization. *A*, Human metaphase hybridized with biotinylated Ham2 DNA. *B*, Distribution of Ham2 DNA over Y chromosomes from 21 cells.

Although there is no direct evidence for X inactivation of the AMGX locus, this is suggested by the mosaic pattern of normal and abnormal enamel formation in female carriers of X-linked AI (Witkop 1967). On the other hand, individuals with one extra X chromosome—e.g., individuals such as 47,XXX females and 47,XXY males—were found to have thicker enamel than do normal males and females, suggesting that the extra X chromosome may be active in amelogenesis (Alvesalo and Portin 1980; Alvesalo et al. 1987). It is also possible that on the X chromosome there are genes other than the AMGX gene which are involved in amelogenesis. Alternatively, larger tooth sizes may reflect higher levels of amelogenin gene expression, since amelogenins are thought to regulate the formation of enamel crystallites. A final formal possibility is that the AMGX locus escapes X inactivation but that the transcriptional activity of the AMGX gene on an inactive X chromosome is significantly reduced. In a female carrier of X-linked AI, such reduced level of amelogenin in the enamel matrix may be insufficient to sustain normal growth of enamel and may explain the mosaic pattern reported. Reduced transcriptional activity from an inactive X chromosome has been demonstrated in the human steroid sulfatase gene (Migeon et al. 1982).

The existence of a Y gene controlling tooth size (i.e., the TSY locus) has been suggested on the basis of tooth measurements in XX males, XY females, and normal controls (Alvesalo and de la Chapelle 1979; Alvesalo and Varrela 1980). It is tempting to suggest that the TSY and the AMGY loci are identical. Using in situ hybridization, we have mapped the AMGY locus to the short arm of the Y chromosome, in the p11.2 region, consistent with the mapping assignment given by Nakahori et al. (1991*b*) using structurally abnormal Y chromosomes. However, Alvesalo and de la Chapelle (1981) have mapped the TSY gene to Yq11 on the basis of a single case in which a male with a deletion of the long arm of the Y chromosome was found to have smaller teeth. Further work is needed to map the TSY gene unambiguously, and the possibility remains that the TSY and the AGMY loci are the same.

The human X and Y chromosomes share significant sequence similarity, likely because of their evolutionary origin from a common ancestral homologous chromosome pair. To date, in addition to the amelogenins, four classes of gene products have been identified which are expressed from both the X and Y chromosomes; these classes include two pseudoautosomal genes—the granulocyte macrophage-colony—stimulating factor receptor (CSF2R) gene (Gough et al. 1990) and the MIC2 gene encoding a cell surface antigen (Goodfellow et al. 1986)—the ZFX/ZFY genes encoding zinc-finger proteins probably involved in the formation of germ cells (Schneider-Gadicke et al.

1989), and the RPS4X/RPS4Y genes encoding two isoforms of ribosomal proteins (Fisher et al. 1990). The MIC2 locus on the X chromosome, the ZFX, and the RPS4X loci have all been shown to escape X inactivation. The CSF2R locus on the X has not been shown to escape X inactivation; however, it is likely to do so, given its pseudoautosomal location. Thus the AMGX/AMGY genes are functionally unique in that they may represent the only X/Y gene pair in which X inactivation may act on the X locus so that males potentially have more gene product than females. The transcriptional products of the X and Y loci are both qualitatively and quantitatively different.

Biochemical characterization of amelogenins has been hampered by the heterogeneity of these proteins. Chromatographic analysis of bovine enamel matrix proteins has revealed a major amelogenin form of 25–30 kD, as well as smaller fragments that seem to originate from postsecretory proteolytic processing of the precursor amelogenin molecule (Termine et al. 1980). These proteins were separated into >100 species by two-dimensional gel electrophoresis (Shimokawa et al. 1987*b*). It has been shown both in cattle and in mouse that there is only one amelogenin gene (Shimokawa et al. 1987*b*; Lau et al. 1989). Although post-translational modifications such as glycosylation and phosphorylation may account for some of the variations in the proteins (Deutsch 1989), the heterogeneity is generated to some degree by alternative splicing of the amelogenin transcripts. In vitro translation of ameloblast mRNA from human (Farge et al. 1987), cow (Shimokawa et al. 1987*b*), and mouse (Slavkin et al. 1982) has revealed multiple proteins recognized by anti-amelogenin antibodies. Since the in vitro translation system minimizes posttranslational modification, multiple proteins are most likely to be generated from multiple mRNA species. Although only a single species of amelogenin mRNA has been detected in mouse (Snead and Lau 1987), two major species of bovine amelogenin mRNA have been identified (Young et al. 1987), and bovine cDNA clones with different amelogenin sequences have been isolated (Shimokawa et al. 1987*a*, 1987*b*). In humans the situation is complicated by the presence of two amelogenin genes on the sex chromosomes. We have isolated three classes of cDNA clones corresponding to alternatively spliced products of the AMGX gene. Although initially we had isolated only one kind of cDNA from the AMGY locus, PCR amplification of the male tooth-bud mRNA by using Y-specific primers indicated that the AMGY transcripts are also alternatively spliced. In addition, there is a transcript unique to the Y gene, suggesting that the AMGX and AMGY transcripts are differentially spliced.

Acknowledgments

We thank Malcolm Snead for pGEM17-2, Hsiao-Fen Huang and Siao-Ping Tsai for excellent technical assistance, Nelva Hitt for help in preparing the manuscript, Sobpheacnea Koy for preparing the illustrations, and T. Mohandas and J. Ellison for useful input and discussion. The work was supported by funds from the Howard Hughes Medical Institute and by National Institutes of Health grant HD12178, March of Dimes grant 1–639, U.S. Department of Energy grant w-7405-ENG-48, and Spain Ministry of Education grant PM-89-0136.

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