Bovine Amelogenin Message Heterogeneity: Alternative Splicing and Y-Chromosomal Gene Transcription†

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ABSTRACT: The amelogenins are the most abundant proteins in developing tooth enamel. Previous analyses have demonstrated that transcriptionally active genes encoding the proteins are located on both the bovine X and the bovine Y chromosomes. We report here the cloning and sequence analysis of the Y-chromosomal gene and corresponding cDNA. The Y-specific mRNA encodes a translation product in which a 21 amino acid domain has been deleted, relative to the X-specific amelogenin, resulting in loss of a structure tentatively described as a β-spiral. There are also 13 single amino acid differences compared to the X-specific amelogenin. In addition, we have cloned and sequenced an X-chromosomal alternatively spliced amelogenin cDNA that encodes a 43 amino acid amelogenin primary translation product. Hydrophobicity analysis indicates that all analyzed amelogenin proteins have a mean hydrophilic character and the two peptides translated from alternatively spliced messages have significant increases in percentage of hydrophobic amino acids.

The enamel proteins are a diverse group of extracellular matrix proteins secreted by ameloblasts during tooth enamel formation. The amelogenins, the most abundant class of proteins, appear to be crucial for proper enamel development, since a deletion in the human X-chromosomal amelogenin gene is associated with the inherited enamel defect amelogenesis imperfecta (Lagerstrom et al., 1991). Mineralization begins as enamel proteins are secreted, and during enamel formation, multiple amelogenin species are present (Termine et al., 1980). Some of these proteins are thought to result from proteolytic processing, which occurs primarily during maturation of enamel [reviewed in Deutsch (1989)], but other explanations, such as multiple mRNAs, have been proposed. While 15–20% of the developing enamel is composed of organic matrix, the protein content of mature enamel is less than 1% (Eastoe, 1963). Most amelogenin protein is lost during the maturation phase, as enamel becomes the most highly mineralized tissue in the body [reviewed in Deutsch (1989)].

The amelogenin genes have been localized exclusively to the bovine and human X and Y chromosomes (Lau et al., 1989), and Southern blot analysis of bovine or human genomic DNA gives a simple banding pattern when probed with amelogenin cDNA (Shimokawa et al., 1987), suggesting that a large gene family does not contribute to protein diversity. The bovine X-chromosomal amelogenin gene was cloned, and DNA sequence analysis indicated that it contains six exons (Gibson et al., 1991b). Most of the X-specific coding regions are in exons 2–5, and one amino acid is encoded by exon 6. A partial Y-chromosomal cDNA has also been cloned and sequenced (Gibson et al., 1991b). This Y-chromosomal cDNA was identified because an oligomer probe, identical to a segment of its 3′-untranslated region, hybridized to male genomic DNA, while X-chromosome-specific amelogenin oligomers hybridized to both male and female genomic DNA (Gibson et al., 1991b).

The amelogenin proteins demonstrate a high degree of conservation at the amino acid sequence level for bovine, mouse, pig, and human species (Fincham et al., 1983; Snead et al., 1985). However, the bovine leucine-rich amelogenin peptide (LRAP) originally identified by Fincham et al. (1983) contains only amino acids 1–33 and 172–184 of the X-specific amelogenin, and we felt that this peptide could result from alternative splicing of the amelogenin primary transcript. This hypothesis was confirmed by cloning and sequencing a bovine amelogenin cDNA from which the coding region for 138 amino acids in exon 5 was deleted by alternative splicing of the primary transcript, and which encoded the LRAP peptide (Gibson et al., 1991a).

In order to better understand the function of the amelogenins, it is necessary to determine the nature of the primary translation products, to distinguish them from the proteolytic breakdown products. To this end, we have analyzed amelogenin cDNAs made from mRNA isolated from bovine developing enamel organs. Through the use of the polymerase chain reaction (PCR), we have identified two additional amelogenin cDNAs, one of which was transcribed from the bovine Y-chromosomal amelogenin gene. Relative to the X-linked amelogenin, the Y-specific predicted translation product has a 21-residue deletion and 13 additional amino acid sequence differences. The other cDNA represents an additional alternative splice product of the X-linked primary transcript. We have also cloned the bovine Y-chromosomal amelogenin gene in order to compare X- and Y-chromosomal amelogenin gene organization and to obtain the complete protein coding sequence.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR) Procedure. The PCR protocol and one of the products was previously described

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(Gibson et al., 1991a). In brief, messenger RNA from ameloblast-rich tissue was copied into cDNA using reverse transcriptase, and 20–60-ng aliquots were amplified by the PCR reaction, using amelogenin-specific oligomers containing *Eco*RI sites at the termini. An aliquot of the PCR product was digested with *Eco*RI and size-separated by electrophoresis in NuSieve GTG agarose (FMC BioProducts, Rockland, ME). Each band containing DNA was excised from the gel, and the DNA was ligated to plasmid vector pUC19 that had been digested with *Eco*RI and calf intestinal alkaline phosphatase. Plasmid DNA from transformed *Escherichia coli* colonies was purified and sequenced by the dideoxynucleotide chain termination procedure (Sanger et al., 1977), either by manual means or by the PCR amplification technique using fluorescent dye-labeled dideoxy terminators, according to the manufacturer's recommendations (Applied Biosystems, Inc., Foster City, CA).

Library Screening. A bovine male genomic library (BL1015j from Clontech Laboratories, Palo Alto, CA) was screened with the 641 base pair *Hin*dIII fragment of the bovine X-specific cDNA (Gibson et al., 1991b). Several positive plaques were isolated and rescreened with X- and Y-specific oligomer probes (Gibson et al., 1991b). One clone that hybridized only to the Y-specific oligomer was analyzed further by restriction enzyme mapping. Fragments of this clone were subcloned into pUC, and the DNA sequence was determined as described above.

Sequence Analyses. Both strands of each cloned DNA insert were sequenced, and data were assembled using the GCG software package (Devereux et al., 1984). Amino acid analysis to determine the degree of conservation was according to the method of Rao (1987), and protein hydrophobicity analysis was according to the Kyte and Doolittle scale (Kyte and Doolittle, 1982).

RESULTS

Characterization of PCR Products. Bovine enamel organ mRNA was copied into single-stranded cDNA, and aliquots were amplified in a PCR reaction. In order to amplify potential alternative splice products as well as Y-chromosome-specific products, sequences for the oligomer primers were chosen near the ends of the known X-chromosome amelogenin-coding regions. The 5′-primer was designed to anneal to nucleotides 59–83 within the signal sequence and the 3′-primer to nucleotides 646–673, a sequence near the end of the translated sequence in exon 5 of the X-chromosomal transcript (Gibson et al., 1991b). Our expectation was that these two regions would be conserved in various amelogenin cDNAs, since there was no evidence for these sequences being involved in alternative splicing.

Four of the observed PCR products were cloned into pUC vectors. The X-specific full-length PCR product, approximately 620 base pairs in length, was identified by comparison to the known sequence (Gibson et al., 1991b), and no further clones derived from this band were analyzed. The Y-specific product was distinguished because of the deletion of 63 nucleotides relative to the X-specific cDNA, as well as single nucleotide differences. We had previously reported a partial Y-specific cDNA sequence isolated by screening a λgt11 cDNA library with an anti-amelogenin antibody probe, that also had this deletion (Gibson et al., 1991b). These new PCR clones yielded 499 base pairs of Y-specific coding region between the two primers. Nucleotide differences between the X and Y PCR products occurring between the primer ends are indicated in Figure 1. There are 36 single-base

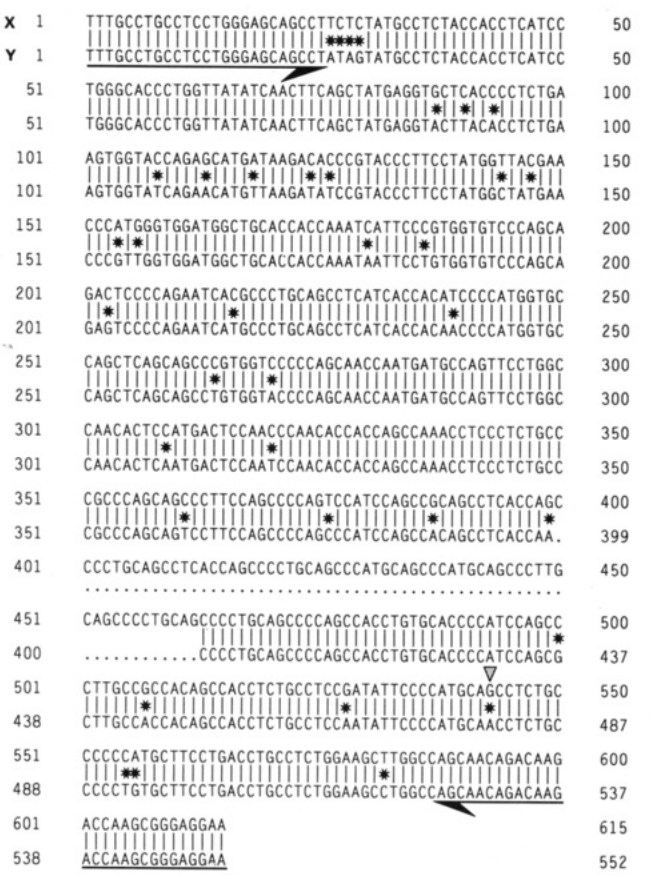

FIGURE 1: DNA sequence of X and Y chromosome-specific cDNA PCR products. Identical nucleotides (|); difference (*). The arrows indicate the position, orientation, and sequence of the PCR oligomer primers. (▼) marks the alternative splice site within the X-specific mRNA.

changes, in addition to the 63-nucleotide deletion (Gibson et al., 1991b).

In addition to the LRAP cDNA previously analyzed (Gibson et al., 1991a), a second series of clones was isolated from among the smallest PCR products. Four clones were sequenced, and each contained the X-specific sequence but lacked exon 3 as well as the coding sequences for amino acids 34–171 within exon 5 (Gibson et al., 1991b). The translated protein, which we will refer to as SAP2, is predicted to contain 43 amino acids, with a molecular weight of approximately 4.7K. Figure 2 provides a comparison for the splicing patterns of the three X-specific mRNAs that have been identified.

Bovine Amelogenin Y-Chromosomal Gene. In order to compare the organization of the X- and Y-chromosomal amelogenin genes and to obtain the full-length Y-specific coding sequence, a bovine male genomic library was screened. A 14 kbp clone subsequently shown to contain the entire Y-chromosomal amelogenin gene was isolated by probing the library with the radiolabeled amelogenin cDNA, followed by a secondary screen using X or Y gene-specific oligomers. The clone was analyzed initially by restriction enzyme mapping, combined with Southern blots probed with exon-specific oligomers. Figure 3 shows a comparison between the structure of the two genes, each of which contains six exons. The coding exons were subcloned, and DNA sequence analysis confirmed the results obtained with the PCR and λgt11 partial cDNA clones and provided additional sequence information required to complete the coding sequence analysis. This provided the sequences in the Y cDNA to which the primers annealed (all PCR products contained X-specific primer sequences), as well as additional 5′ and 3′ sequence information. There was a

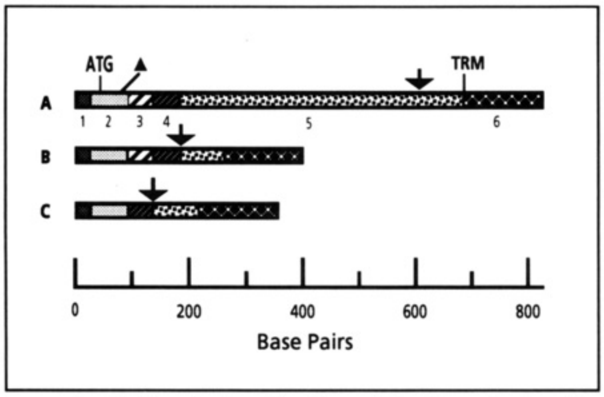

FIGURE 2: Bovine amelogenin X-specific alternatively spliced mRNAs. Exons are numbered 1–6; ATG and TRM are the translation initiation and termination sites; (▲) is the N-terminus after the signal secretion signal has been cleaved; (↓) marks the position of the alternative splice site within exon 5. (A) Full-length amelogenin, 197 amino acids; (B) leucine-rich amelogenin peptide (LRAP), 59 amino acids; (C) X-chromosome-specific peptide, SAP2, described in present paper, 43 amino acids.

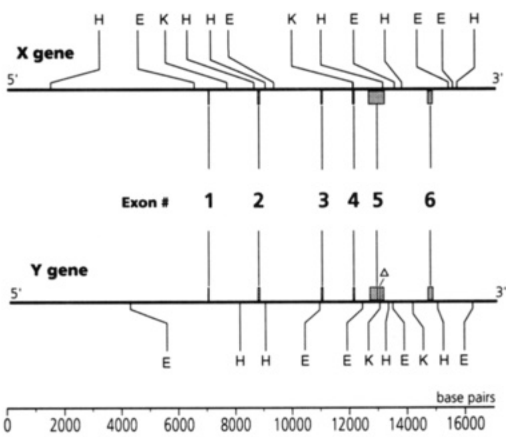

FIGURE 3: Structure of the bovine amelogenin X- and Y-chromosomal genes. Exons are numbered 1–6; (△) indicates the deletion in exon 5 of the Y-chromosomal gene. The position of exon 1 in the Y-chromosomal gene was estimated by restriction enzyme mapping. E = *Eco*RI; H = *Hin*dIII; K = *Kpn*I.

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X   1  MGTWILFACLLGAAFSMPLPPHPGHPGYINFSYEVLTPLKWYQSMIRHPY   50
       |||||||||||| |•||||||||||||||||||||||||||||•|•| ||
Y   1  MGTWILFACLLGGAYSMPLPPHPGHPGYINFSYEVLTPLKWYQNMLRYPY   50
                       2-3              3-4           4-5

   51  PSYGYEPMGGWLHHQIIPVVSQQTPQNHALQPHHHIPMVPAQQPVVPQQP  100
       ||||||| |||||||||||||||•||||||||||| ||||||||||||||
   51  PSYGYEPVGGWLHHQIIPVVSQQSPQNHALQPHHHNPMVPAQQPVVPQQP  100

  101  MMPVPGQHSMTPTQHHQPNLPLPAQQPFQPQSIQPQPHQPLQPHQPLQPM  150
       ||||||||||||•|||||||||||||•||||•|||||||
  101  MMPVPGQHSMTPIQHHQPNLPLPAQQSFQPQPIQPQPHQ...........  139

  151  QPMQPLQPLQPLQPQPPVHPIQPLPPQPPLPPIFPMQPLPPMLPDLPLEA  200
                 |||||||||||| |||||||||||||||||| ||||||||
  140  ..........PLQPQPPVHPIQRLPPQPPLPPIFPMQPLPPVLPDLPLEA  179

  201  WPATDKTKREEVD                                        213
       |||||||||||||
  180  WPATDKTKREEVD                                        192
                 5-6
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FIGURE 4: Predicted amino acid sequence of bovine X- and Y-specific amelogenins. The signal secretion signal is encoded by amino acids 1–16. Vertical lines indicate identity; conserved amino acids are marked by (●), and nonconserved amino acid differences are blank. Numbers beneath the lines represent exon boundaries.

Table I: Analysis of Amelogenin Hydrophobicity

amelogenin	hydrophobicity[a] mean	% of residues[b]	sequence length
bovine X	–0.738	27.41	197
bovine Y	–0.728	27.27	176
human X	–0.706	26.14	176
human Y	–0.663	27.68	177
mouse	–0.715	27.22	180
pig	–0.729	27.17	173
bovine LRAP	–0.622[c]	28.81[d]	59
bovine SAP2	–0.553[c]	32.56[e]	43

[a] Hydrophobicity was calculated using the values of Kyte and Doolittle (1982). The scale ranges from 4.5 for the most hydrophobic amino acid (Ile) to –4.5 for the most hydrophilic (Arg). [b] Computed as the percentage of all residues which are hydrophobic. Hydrophobic residues include Ala, Cys, Ile, Leu, Met, Phe, and Val. [c] Significantly different from the mean hydrophobicity of all full-length species (–0.713); $p < 0.001$. [d] Significantly different from the mean percent hydrophobic residues of all full-length species (27.15); $p < 0.002$. [e] Significantly different from the mean percent hydrophobic residues of all full-length species (27.15); $p < 0.001$.

one-nucleotide difference at position 507 from that previously reported (Gibson et al., 1991b), which may be attributable to sequence heterogeneity or error. This difference was in the "wobble" position and does not alter the predicted amino acid sequence. At each splice position, the required GT and AG dinucleotides (Mount, 1982) are present at the boundaries of the introns. The five nucleotides adjacent to each dinucleotide are identical to the corresponding sequences in the X-chromosomal gene (Gibson et al., 1991b) except for three, two of which have C in the Y sequences in place of T in the X, and the third with one A to G difference.

Differences observed between bovine X and Y-specific gene sequences can be summarized as follows: (1) There are 41 single-nucleotide differences between the X and Y gene-coding regions. (2) There are a total of 13 amino acid differences including 6 conservative and 5 nonconservative changes in the secreted protein (Rao, 1987) plus 2 differences in the signal sequence; Figure 4 presents the amino acid sequence comparisons. (3) Sixty-three nucleotides have been deleted from the Y gene exon 5, which would result in the loss of 21 amino acids from the translated protein. (4) The 3′-untranslated regions have little homology.

Amelogenin Hydrophobicity. The amelogenins have been described as hydrophobic proteins rich in proline, histidine, leucine, and glutamine, but with a hydrophilic carboxy terminus [see Deutsch (1989) for a review]. A hydrophobic nature was suggested partly due to early experiments in which bovine enamel proteins showed temperature-dependent aggregation characteristic of hydrophobic interactions (Nikiforuk & Simmons, 1965). In order to better understand whether amino acid differences might contribute to alterations in protein hydrophobicity, amelogenin proteins were analyzed by the Kyte and Doolittle scale (Kyte & Doolittle, 1982). The data in Table I show that the amino acid composition of the various amelogenins has a mean hydrophilic character and that hydrophobic residues are present in about the same proportion in all of the known full-length sequences. Both LRAP and SAP2, the products of alternatively spliced bovine mRNAs, are significantly more hydrophobic than the longer sequences.

DISCUSSION

The amelogenin proteins are known to be conserved in diverse species (Herold et al., 1987) and therefore are likely to perform a specialized function during enamel formation. The bovine X- and Y-chromosomal genes are structurally similar, introns are placed at identical positions within each gene, and splice consensus sequences are similar. Ninety-three percent of the nucleotides are identical within the coding regions of the 2 genes, and the N-terminal 27 amino acids are also identical to each other and to human and porcine amelogenins (Fincham et al., 1983). Partial human X- and Y-

specific exon sequences also have 93% homology within the coding regions (Nakahori et al., 1991). In addition, the bovine Y-chromosomal locus is active because Y-specific transcripts can be demonstrated.

The most striking difference between the X- and Y-chromosomal amelogenin genes is the 63 base pair deletion in exon 5 of the Y-linked gene. The 21 encoded amino acids in the X-specific amelogenin are part of a unique structure tentatively described as a β-spiral (Renugopalakrishnan et al., 1986), which contains a proline at every third residue. Interestingly, this domain is not present in the human X- or Y-chromosomal genes (Nakahori et al., 1991) nor in mouse cDNA (Snead et al., 1985), and its function is unknown. In addition, 13 of the 192 amino acids are different with respect to the X-specific protein, and only 5 changes are considered to be nonconservative, 1 in exon 4 and 4 in exon 5. Although the amelogenins have been described as hydrophobic proteins, the calculated character, according to Kyte and Doolittle analysis (Kyte & Doolittle, 1982), is hydrophilic, and it is unaffected by amino acid differences in X and Y chromosome-specific full-length amelogenins. Attempts to demonstrate periodic clustering of hydrophobicity anywhere in the amelogenin proteins did not reveal significant arrays of residues (not shown), with the exception of the QPX (where X = L, H, or M) repeat in the bovine X-specific gene product. Taken together, these analyses suggest that the arrangement and content of hydrophobic residues in amelogenins are not sufficient either to explain their apparent hydrophobic properties (Nikiforuk & Simmons, 1965) or to provide clues to their biologic function.

The additional alternatively spliced X-specific cDNA does not encode amino acids 3–18 in exon 3, nor amino acids 34–171 within exon 5, which also deletes the repetitive β-spiral domain. Hydrophobicity analysis revealed that both LRAP and SAP2 are significantly more hydrophobic than the longer sequences, which may have effects on protein function. A question that arises is what could be the role for these small amelogenin peptides during enamel formation. All amelogenin proteins that have not been subjected to proteolytic cleavage have identical sequences for the first 27 amino acids, with the exception of a porcine amino acid sequence which is likely to have been produced by splicing out only exon 3, since its mass was reported as 18 kDa (Yamakoshi et al., 1989). When amino- and carboxyl-terminal amelogenin peptides were individually tested in vitro for the capacity to inhibit hydroxyapatite crystal formation, neither peptide had significant activity compared to the full-length protein (Aoba, 1989). Partially degraded porcine amelogenin peptides also had decreased activity, supporting the conclusion that the entire protein is necessary for this activity to be observed (Aoba et al., 1987). Activity of the LRAP peptide, which contains both the amino- and carboxy-terminal amelogenin domains, has not been reported. Perhaps the key to understanding the role of the smallest amelogenin primary translation products is that they both contain the exon 4 coding sequence (Figure 2), and although this domain is not as highly conserved as the amino terminus, it may impart a function crucial to the protein's activity, as it is present in all amelogenins described to date.

Alternative splicing has been detected only in the X-chromosomal primary transcript, and in both cases, a site within exon 5 is used as splice acceptor. We have not observed Y-specific alternative splicing, and we theorize that it is because the CAG codon at residue 171 in the X transcript has been altered to a CAA codon in the Y transcript (Figure 1). This change would remove the consensus AG (Mount, 1982) in exon 5 required for splicing of the X-specific primary transcript.

A major unresolved question is whether the Y-specific transcript and the small X-specific alternatively spliced message are translated. We have shown that X and Y gene structures are similar and that a Y-specific transcript is produced which is properly spliced. Although the Y gene does not have a consensus polyadenylation signal, there is an ATTAAA sequence 22 bases upstream from the 3′ end of the mRNA which may be performing this function and the Y-specific mRNA is polyadenylated (Gibson et al., 1991b). There is an appropriate open reading frame, and the sequence surrounding the proposed ATG start codon is similar in the two genes. Therefore, there is no apparent defect in the message that suggests it would not be translated. In addition, differences have been reported between enamel proteins isolated from developing human male and female teeth which may indicate that the Y-chromosomal gene is actively transcribed and translated (Fincham et al., 1991).

We can formulate the following hypotheses to explain differences in the bovine amelogenin proteins. (1) The X- and Y-specific proteins carry out identical functions, and amino acid differences are in areas of the proteins that are not crucial for those functions. (2) The Y-specific protein has a somewhat different function, only required for formation of enamel in male animals. The proteins translated from alternatively spliced transcripts have lost the β-spiral, and the smallest also has lost the exon 3 domain; both of these smaller proteins are likely to have functions distinct from the full-length amelogenins. We are currently testing potential functions of the various amelogenin proteins by in vitro experiments.

In humans, the amelogenin genes have been localized to Xp22.1-22.3 and to the Y chromosome, tentatively to Yq11 (Lau et al., 1989). Genes with loci only on the X chromosome are usually subject to inactivation early in development in females [reviewed by Lyon (1988)]. This is a dosage compensation mechanism which results in an equivalent amount of gene product being produced in female cells with two X chromosomes as compared to male cells with only one X chromosome. Female patients with hereditary X-linked amelogenesis imperfecta have enamel that has a vertical stripe pattern of good and poor enamel. This suggests that the amelogenin gene is indeed subject to X inactivation and in these patients adjacent groups of ameloblasts secreted either the normal or the defective amelogenin product (Witkop, 1967). However, it has been observed that genes with both X- and Y-chromosomal loci generally escape X inactivation (Schneider-Gadicke et al., 1989). If the X-linked amelogenin gene proves to be X-inactivated in females, while both loci are active in males, this unique feature would make the amelogenin genes distinct from other sex-chromosomal loci.

Since neither the X nor the Y chromosome in a normal male is inactivated, male ameloblasts may be expected to produce twice as much amelogenin as female ameloblasts. Perhaps the additional protein that may be produced in males can help to explain the observation that male teeth are on average larger than teeth from females (Alvesalo & Portin, 1980). In addition, patients with abnormal numbers of sex chromosomes have alterations in enamel thickness. For example, 47,XYY males (Alvesalo & Tammisalo, 1985), 47,-XXX females (Alvesalo et al., 1987), and 47,XXY males (Alvesalo et al., 1991) all have measurable increases in enamel thickness, supporting the idea that amelogenesis is affected by both X- and Y-chromosomal genes.

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