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Evolution of the Bovine Lysozyme Gene Family: Changes in Gene Expression and Reversion of Function

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Abstract. Recruitment of lysozyme to a digestive function in ruminant artiodactyls is associated with amplification of the gene. At least four of the approximately ten genes are expressed in the stomach, and several are expressed in nonstomach tissues. Characterization of additional lysozymelike sequences in the bovine genome has identified most, if not all, of the members of this gene family. There are at least six stomachlike lysozyme genes, two of which are pseudogenes. The stomach lysozyme pseudogenes show a pattern of concerted evolution similar to that of the functional stomach genes. At least four nonstomach lysozyme genes exist. The nonstomach lysozyme genes are not monophyletic. A gene encoding a tracheal lysozyme was isolated, and the stomach lysozyme of advanced ruminants was found to be more closely related to the tracheal lysozyme than to the stomach lysozyme of the camel or other nonstomach lysozyme genes of ruminants. The tracheal lysozyme shares with stomach lysozymes of advanced ruminants the deletion of amino acid 103, and several other adaptive sequence characteristics of stomach lysozymes. I suggest here that tracheal lysozyme has reverted from a functional stomach lysozyme. Tracheal lysozyme then represents a second instance of a change in lysozyme gene expression and function within ruminants.

Key words: Multigene family — Gene duplication — Concerted evolution — Regulatory evolution — Adaptive evolution — Pseudogenes

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Introduction

Ruminant animals have evolved a new digestive enzyme that has allowed them to exploit plant materials as a food source. (See Irwin et al. 1992 for review.) The new digestive enzyme is a lysozyme *c*, which in mammals typically has an antibacterial function in host defense (Dobson et al. 1984; Jollès et al. 1984). Recruitment of lysozyme to its new role as a digestive enzyme involved adapting the amino acid sequence such that the enzyme could function in the acidic stomach environment, as well as a change in the regulation of the gene so that it could be expressed at high levels in the stomach (Dobson et al. 1984; Jollès et al. 1984, 1989, 1990; Stewart and Wilson 1987; Stewart et al. 1987; Swanson et al. 1991; Irwin et al. 1992). Lysozyme has been recruited as a digestive enzyme by two groups of ruminant mammals—the leaf-eating monkeys (e.g., langur monkey) and the ruminant artiodactyls (e.g., cow). In the ruminant artiodactyls, the recruitment of lysozyme to its new function is associated with duplication of the gene (Dobson et al. 1984; Irwin et al. 1989; Irwin and Wilson 1989), while in the leaf-eating monkeys it is not (Stewart et al. 1987; Swanson et al. 1991).

Advanced ruminants (e.g., cow, sheep, and deer) have approximately ten lysozymelike sequences in their genome (Irwin and Wilson 1989; Irwin et al. 1989). Cow stomachs were found to contain three different lysozymes (Dobson et al. 1984), and these enzymes are encoded by a minimum of four genes, two of which are nearly identical (Irwin and Wilson 1989). Genes for the three cow stomach lysozymes have been characterized

(Irwin et al. 1993). Lysozyme is also expressed in other tissues of the cow. At least three lysozyme genes are expressed in tracheal tissue, though one encodes a cDNA, clone 7a, which appears to be an allele of cow stomach 2 lysozyme gene (Takeuchi et al. 1993). The predicted amino acid sequence of another of the tracheal cDNAs, clone 5a (Takeuchi et al. 1993), is identical to the amino acid sequence determined from lysozyme purified from the kidney (Ito et al. 1993). A cDNA identical to the tracheal cDNA 5a was also isolated from neutrophil granulocytes and mammary gland tissue (Steinhoff et al. 1994). The lysozyme purified from kidney appears to be widely expressed in nonstomach tissues. Along with the complete sequence of sheep kidney lysozyme (Ito et al. 1993), partial amino acid sequences are known for a few additional nonstomach ruminant lysozymes—namely, a cow milk (White et al. 1988) and three goat tear (Jollès et al. 1990) lysozymes.

Here I report the characterization of five additional lysozymelike genomic sequences of the cow. A genomic clone was characterized that contained a sequence very similar to one of the cDNAs (cow 14d) expressed in tracheal tissue (Takeuchi et al. 1993), and it probably represents the functional gene. A second clone contained a pseudogene that may be a descendant of one of the earliest duplications of the lysozyme gene on the ruminant lineage. Fragments of two stomachlike lysozyme pseudogenes were characterized, and from them it could be inferred that concerted evolution involves more than four of the lysozyme genes. Amplification by PCR indicated the presence of a second gene related to the gene that encodes the kidney lysozyme. No evidence of recombination between stomach and expressed nonstomach lysozyme genes, or among the nonstomach lysozyme genes, was found. These results suggest that all, or nearly all, of the cow lysozymelike genes have now been identified.

Materials and Methods

Isolation and Characterization of Lysozyme Genes. A total of 18 bovine (*Bos taurus*) lysozyme genomic clones had been previously isolated, 13 of which encoded stomach lysozyme genes (Irwin et al. 1993). Portions of the five remaining lambda genomic clones were subcloned into Bluescript KS^+ or pUC13 and sequenced as previously described (Irwin et al. 1993). A portion of exon 2 of the kidney type of lysozyme genes was amplified by PCR (Saiki et al. 1988) using degenerate primers designed on the basis of conserved portions of the sheep and cow kidney lysozyme amino acid sequences (Ito et al. 1993) that differed from the stomach sequences (Jollès et al. 1989). The primer sequences and locations are: 5′ sense primer, amino acids 28–33, 5′-CCGAATTCGGGATGTGYYTNGCN(C/A)G-3′, and the 3′ antisense primer, amino acids 69–64, 5′-GCGGATCCTTNCCRTCRTTRCACCA-3′, where N is any nucleotide, Y is C or T, and R is A or G. Genomic DNAs used in the amplifications were as previously described (Irwin et al. 1989, 1991). PCR products were separated in polyacrylamide gels and cloned into M13 mp19 for sequencing by standard methods (Sambrook et al. 1989).

DNA Sequence Analysis. Lysozyme gene DNA sequence data were managed and assembled using the MacDNASIS (Hitachi) software package. The DNA sequences reported in this paper have been deposited into the GenBank data base under the following accession numbers: U19466 and U19467 for the complete gene sequences of trachea (clone 208) and ΨNS4 (clone 205), U19468 and U19469 for the partial gene sequences of ΨS4 (clone 217) and ΨS5 (clone 222), and U19470–U19480 for the amplified portions of the kidneylike lysozyme sequences from cow (4 sequences, including cow NS3), goat (3 sequences), sheep (3 sequences), and fallow deer (2 sequences).

Phylogenetic Analysis. Phylogenetic trees were derived from both amino acid and gene sequences of lysozymes. For phylogenetic analysis two DNA sequence alignments were created: an alignment of the coding sequences corresponding to mature lysozymes and an alignment of gene sequences. The coding alignment included the sequences from cow, sheep, and deer stomach cDNAs (Irwin and Wilson 1989, 1990), cow tracheal cDNAs (Takeuchi et al. 1993), the human gene (Peters et al. 1989), primate cDNAs (Swanson et al. 1991), and rodent genes (Cross et al. 1988; Yeh et al. 1993), as well as the sequences reported here. Sequence alignment of the coding region was by hand, with gaps introduced to maximize alignment and to maintain the reading frame. The alignment of the gene sequences included the 5′ flanking, exon, and intron sequences of the cow stomach genes (Irwin et al. 1993), the human gene (Peters et al. 1989), and those reported here. Flanking and intron sequences of rodent genes could not be reliably aligned with the other sequences. Gene sequences were aligned manually with the guidance of alignments derived from a dot-plot analysis conducted with MacDNASIS. In the dot-plot analysis, a dot indicates that in a window of ten bases the two sequences were identical for at least eight positions. For the analysis of the genomic sequences, all repetitive DNAs were first removed, and then distances were estimated from flanking and intron regions not found to participate in concerted evolution of the cow stomach genes (Irwin et al. 1993). Distances were then estimated after deleting any position that had a gap in any sequence.

Neighbor-joining trees of lysozyme sequences were calculated using the MEGA software package (Kumar et al. 1993). Synonymous and nonsynonymous divergences of coding regions of mature lysozyme and divergence of intron and flanking sequences were calculated after deleting nucleotide positions which had a gap in any sequence (complete-deletion option) and corrected by the Jukes and Cantor method for coding sequences or the Kimura two-parameter method for noncoding sequences. Bootstrapped neighbor-joining trees were constructed from distances estimated from 500 bootstrap replications of the sequence data. Parsimony trees were constructed using PAUP 3.1.1 (Swofford 1993) with bases considered as unordered character-state data for the DNA sequence alignments and a genetic code stepmatrix (PROTPARS) was used for the amino acid sequences. Gaps were considered as missing data. Trees were identified using a heuristic search with random addition of taxa (ten replications) and bootstrapping of the data 500 times.

Results

Characterization of Four Cow-Lysozymelike Genes

A previous screening of two cow genomic libraries resulted in the isolation of 18 genomic clones, 13 of which encoded stomach lysozyme genes plus 5 others (Irwin et al. 1993). The five genomic clones that did not encode stomach lysozymes are characterized here; they encode four lysozymelike sequences (a second clone overlapped with clone 217 encoding ΨS4) which are summarized in Fig. 1. Complete gene sequences (four exons and in-

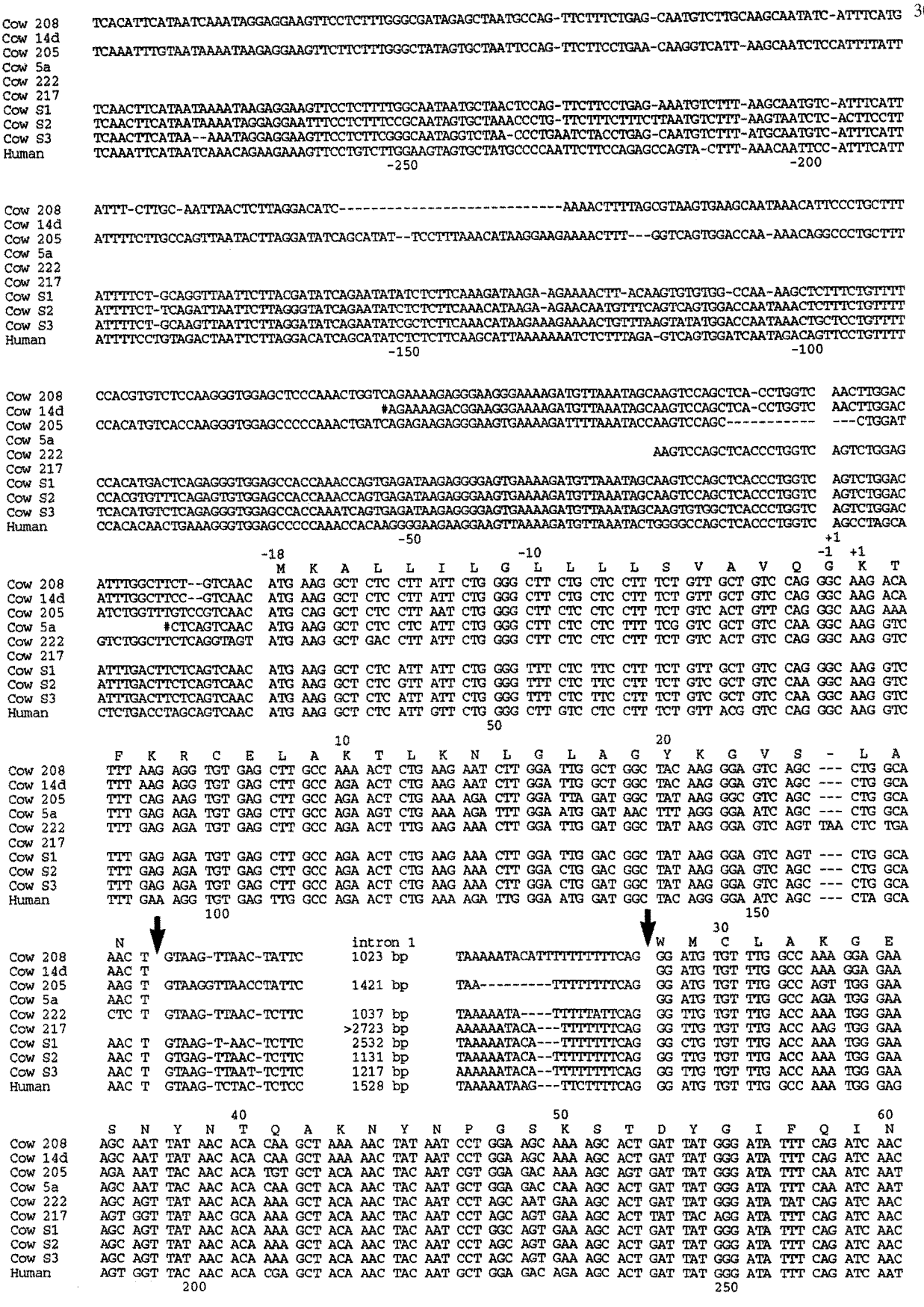

Fig. 1. Sequences of the cow lysozyme genes. Alignment of the cow and human lysozyme genes is summarized. *Cow S1, S2,* and *S3* are from Irwin et al. (1993), trachea (clone *14d*) and kidney (clone *5a*) from Takeuchi et al. (1993), and the *human* gene from Peters et al. (1989). The predicted amino acid sequence of clone *208* is shown above in the single-letter amino acid code, and is *numbered* from the N-terminal of the mature protein. The gene sequences are *numbered* from the mRNA start site of the human and cow stomach genes, with intron and repetitive (in the 3′ untranslated region) sequences omitted. Intron–exon junctions are indicated by the *vertical arrows.* The promoter and 5′ flanking sequences are *numbered backwards* from the mRNA start site. The site of polyadenylation of the human and cow stomach genes is indicated by the diamond, with a consensus polyadenylation signal *underlined* (bases 1342–1347). Gaps introduced for alignment are shown as *dashes.* Gaps due to the absence of characterized sequences (e.g., introns for clones 5a and 14d) appear as *blanks.* the 5′ ends of cDNAs (5a and 14d) are indicated by #. Repetitive DNA elements found in the 3′ untranslated portion of the human and cow 205, 5a, and 14d sequences have been omitted where indicated (<<< >>>).

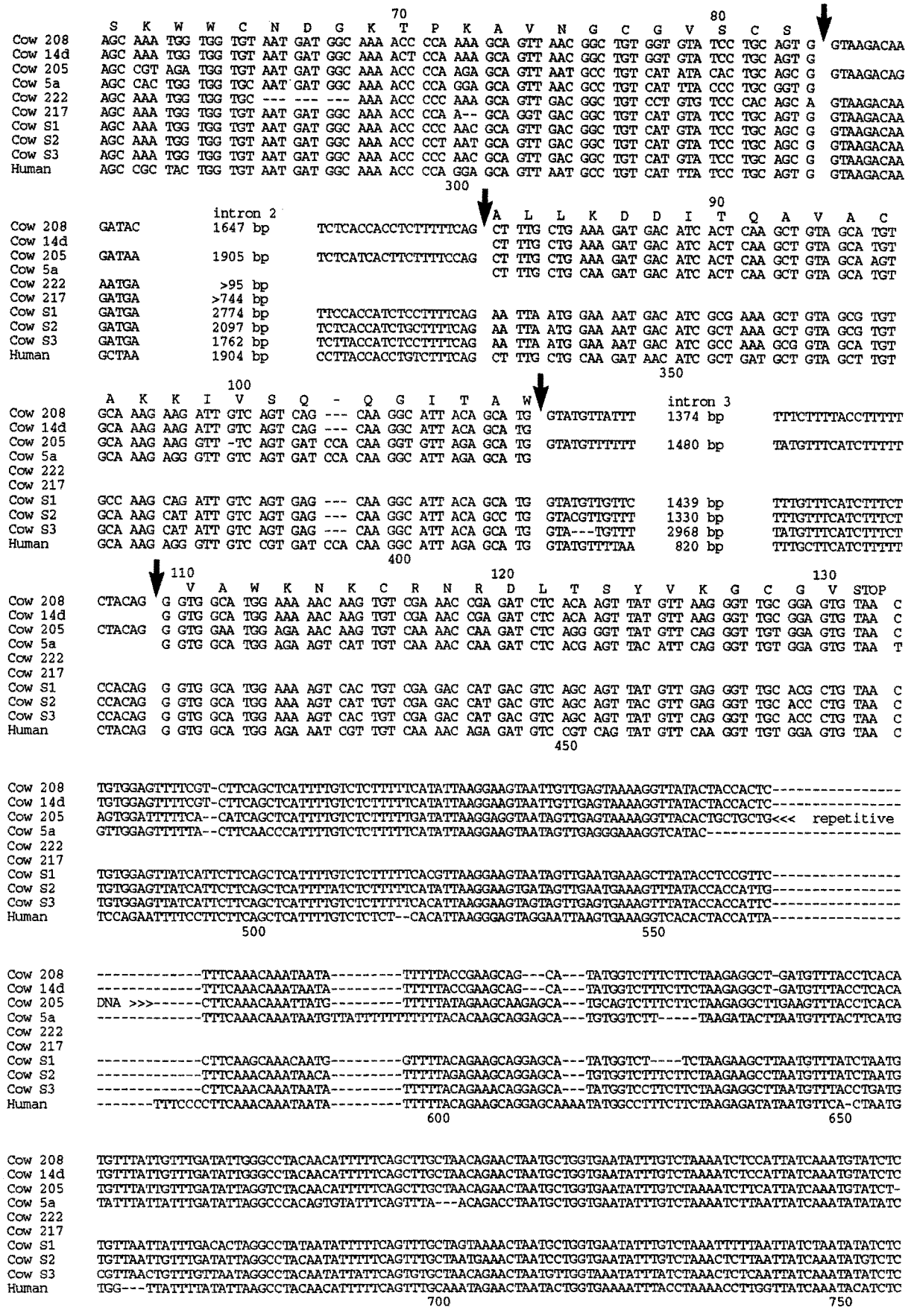

Fig. 1. Continued.

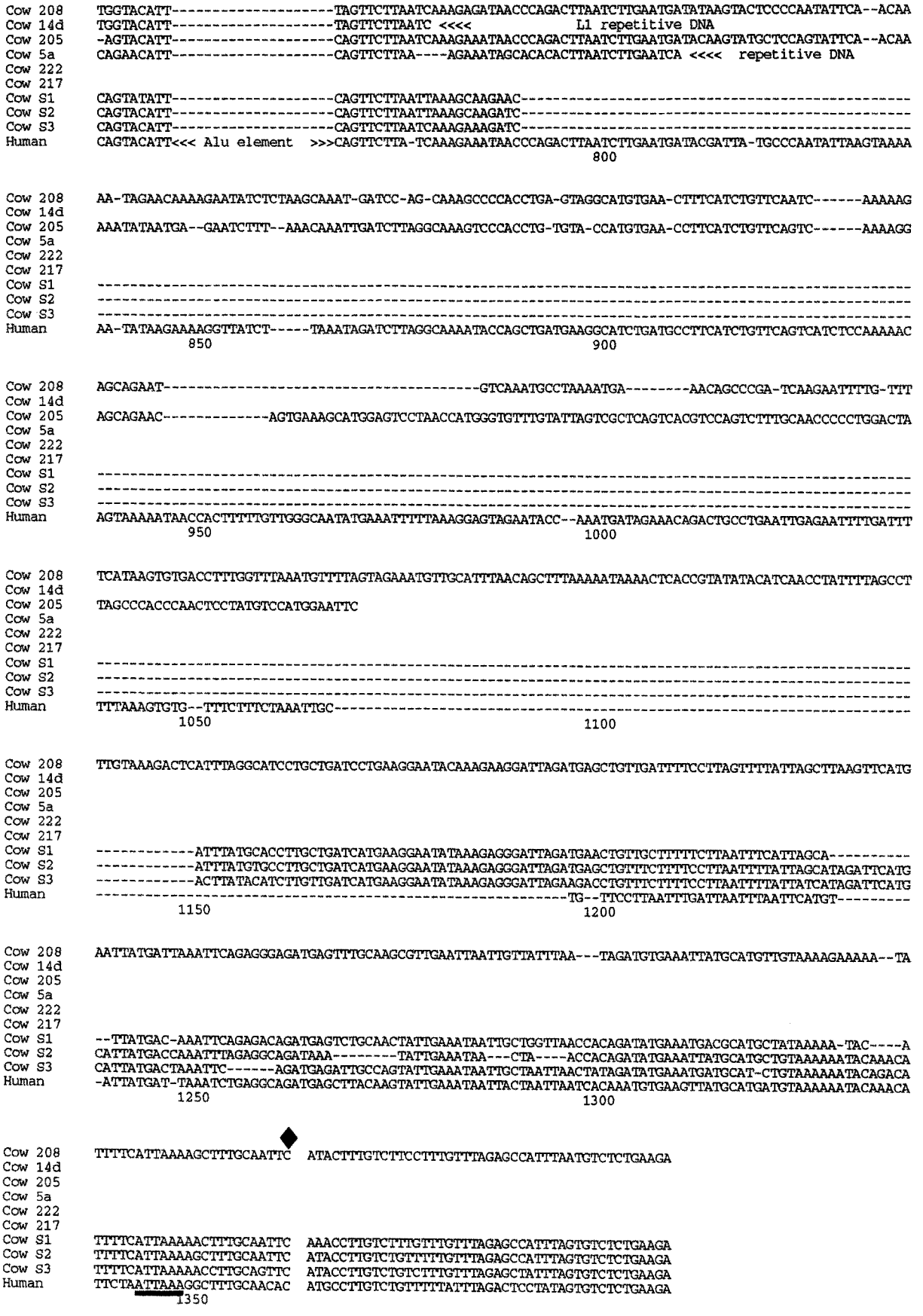

Fig. 1. Continued.

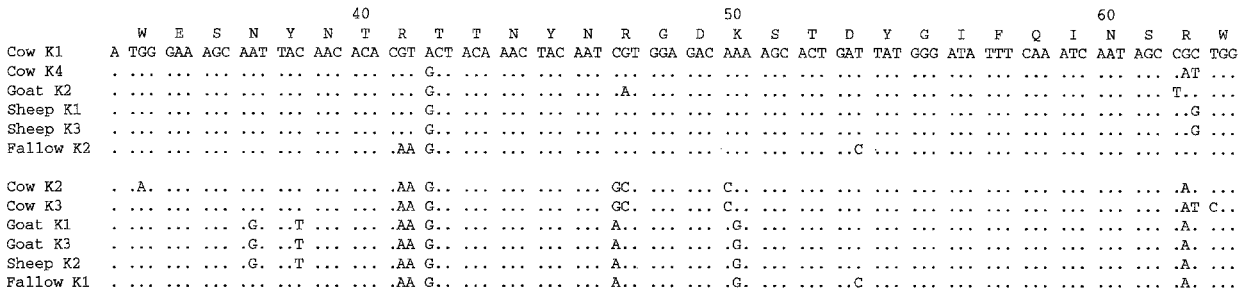

Fig. 2. Sequences of lysozyme sequences amplified via PCR. DNA sequences of cloned PCR products amplified with primers described in the text are presented. The predicted amino acid sequence of residues 34–63 of cow clone *K1* is shown above the DNA sequence, with *numbering* according to the mature protein indicated. The positions of the amplification primers are immediately adjacent to the presented sequences.

trons) were obtained for two of the bovine lysozyme genes, ΨNS4 and trachea, while the sequence of only one or two exons could be determined for ΨS4 (exon 2) and ΨS5 (exons 1 and 2) (Fig. 1). A total of 8,942 bases of trachea gene, 8,789 bases of ΨNS4, 3,264 bases of ΨS4, and 1,522 bases of ΨS5 were determined. The lengths, from predicted mRNA start site to polyadenylation site (Fig. 1), of the ΨNS4 (>6,197 bp) and trachea (5409 bp) genes, are similar to those of other cow lysozyme genes (Irwin et al. 1993).

Sequences found in the clones 217 (ΨS4), 222 (ΨS5), and 205 (ΨNS4) appear to be pseudogenes. As shown in Fig. 1, clone 217 (ΨS4) has two basepair deletions (bases 300–301) resulting in an in-frame stop codon (bases 307–309) in exon 2; clone 222 (ΨS5) has two in-frame stop codons (between amino acid residues 24 and 25, bases 155–157, and at residue 26, bases 161–163) in exon 1; and clone 205 (ΨNS4) has a single base deletion (base 383) which results in a frame shift and replacement of all amino acid residues beyond amino acid residue 99. In addition there are several other unexpected amino acid substitutions in each of the sequences, including the replacement of conserved cysteine residues in ΨS5 (bases 326–328, cysteine^{81} to histidine) and ΨNS4 (bases 368–370, cysteine^{95} to serine), and deletions, including deletion of the mRNA start site in ΨNS4 (bases −11 to +3) and three amino acids in ΨS4 (bases 281–289, amino acids 66–68) (see Fig. 1). The exon sequences of ΨS4 and ΨS5 were most similar to other cow stomach lysozyme cDNAs (Irwin and Wilson 1989). ΨNS4 is approximately equally different from stomach (S1, S2, S3) (Irwin and Wilson 1989) and from either of the two nonstomach lysozyme sequences (trachea and kidney) (Takeuchi et al. 1993; Steinhoff et al. 1994).

The gene encoded by genomic clone 208 (tracheal lysozyme) is likely to be a functional gene because it has only one amino acid difference from that predicted from the tracheal cDNA 14d, at residue 10 of the mature protein (Fig. 1). Comparison to other lysozyme gene and cDNA sequences showed that the exons of clone 208 are very similar in sequence to the sequence of the cDNA 14d isolated from tracheal tissue (Takeuchi et al. 1993): There are only three additional silent differences between base −52 and base 777 of the cDNA sequences (Fig. 1). cDNA 14d and clone 208 differ most strikingly in that the cDNA 14d contains an L1-like repetitive DNA sequence (>446 bp) inserted downstream of base 777 that is absent in clone 208. Genomic clone 208 and cDNA 14d either represent two alleles of a single gene, or tracheal lysozyme, or two very closely related genes.

Amplification and Isolation of Kidneylike Lysozyme Sequences

Partial amino acid sequences were available for several lysozymes expressed in nonstomach tissues, and it was suspected that there could be a subfamily of nonstomach lysozyme genes (Jollès et al. 1990; Ito et al. 1993). Degenerate PCR primers specific for kidney lysozymes were designed which should not (and do not) amplify genes encoding ruminant stomach or trachea (clone 208) sequences. Amplifications resulted in product(s) from cow, sheep, goat, fallow deer, and camel, but not from pig, zebra, rhesus monkey, or elephant (data not shown). To permit identification of possible multiple sequences, the PCR products were cloned and sequenced. Multiple sequences were obtained from each species, though many of the differences may be due to errors introduced during PCR (Irwin and Wilson 1990). The sequences from cow, sheep, goat, and fallow deer (Fig. 2) suggest the existence of at least two gene sequences. Some of the sequences are nearly identical to the kidney sequence (Takeuchi et al. 1993; Ito et al. 1993; Steinhoff et al. 1994); they are cow clones K2, K3, goat clones K1, K3, sheep clone K2, and fallow clone K1 (Fig. 2). The second type of sequence, cow NS3-like, includes cow clones K1, K4, goat clone K2, sheep clones K1, K3, and fallow clone K2 (Fig. 2).

Discussion

Number of Lysozyme Genes

Genomic Southern blot analysis suggested that the genomes of advanced ruminants, such as cow, sheep, and deer, have approximately ten lysozymelike genes (Irwin

Table 1. Summary of cow lysozyme genes and their expression

Gene (clone)[a]	Site(s) of expression	Sequence evidence from		
		Gene	cDNA	Protein
Stomach				
S1	Stomach	+	+	–[g]
S2[b]	Stomach/trachea	+	+[b]	+
S3	Stomach	+	+	–[g]
ψS4 (217)	—[f]	+	–	–
ψS5 (222)	—	+	–	–
Non-stomach				
Trachea (208)[c]	Trachea	+	+	–[g]
Kidney[d]	Macrophage/trachea/ mammary gland	+[h]	+	+
NS3 (K1)[e]	Not determined	+[h]	–	–
ψNS4 (205)	—[f]	+	–	–
Milk[e]	Mammary gland	–	–	+

[a] Clones described in this paper
[b] Characterization of cDNAs indicate there are two S2 genes (Irwin and Wilson 1989)
[c] Also represented as a cDNA (Takeuchi et al. 1993)
[d] Represented by cDNA clones from Takeuchi et al. (1993) and Steinoff et al. (1994)
[e] Lysozymes NS3 and milk may represent the same gene (see text)
[f] These genes appear to be pseudogenes and if expressed would encode nonfunctional lysozymes
[g] Proteins identified for these genes on basis of electrophoretic mobility, and for S1 and S3, amino acid composition, and immunological comparison (see Dobson et al. 1984; Jollès et al. 1984; Irwin and Wilson 1989; Takeuchi et al. 1993)
[h] From PCR amplification of part of exon 2

Table 2. Percent synonymous divergence of partial sequences of lysozyme genes

Sequences compared	ψS4[a]	ψS5[b]	NS3[c]
Stomach lysozymes			
Cow S1	18.3	11.4	42.3
Cow S2	14.6	16.6	38.3
Cow S3	14.6	11.4	38.3
Sheep S1	22.8	16.4	42.3
Sheep S2	22.8	19.1	42.3
Sheep S3	22.5	16.4	41.2
Deer S1	37.5	16.4	53.5
Deer S2	27.4	21.9	66.6
Nonstomach lysozymes			
Trachea (208)	24.9	32.6	41.8
Trachea (14d)	29.6	35.7	41.8
ψNS4	37.6	45.5	5.9
Kidney	34.7	27.2	9.4
Human lysozyme	32.8	60.7	32.4

[a] Divergence estimated from exon 2 (see Fig. 1)
[b] Divergence estimated for coding regions in exons 1 and 2 that encode the mature protein (see Fig. 1)
[c] Divergence estimated from amplified region of exon 2 (see Fig. 2)

et al. 1989; Irwin and Wilson 1989). Isolation of bovine lysozyme cDNA clones indicates that at least six genes are expressed (Table 1). Clones isolated from a bovine stomach cDNA library showed that a minimum of four genes are expressed in the stomach (Irwin and Wilson 1989). A total of three lysozyme cDNA clones have been isolated from a bovine tracheal cDNA library, but one of these clones appears to be an allele of the cow stomach 2 genes (Takeuchi et al. 1993).

The genomic clones described here encode four lysozymelike genes, one of which, clone 208, may be an allele of a tracheal cDNA 14d (Takeuchi et al. 1993). The predicted size of the mRNA encoded by clone 208 is approximately 1,300 bases, similar in size to that of the mRNA detected by Northern blotting of tracheal cell mRNA with a probe specific for cDNA 14d (and 208) (Takeuchi et al. 1993). These data suggest that clone 208 and cDNA 14d are alleles of one gene—trachea lysozyme. The difference in the 3′ ends of the two alleles is probably due to the recent insertion of an L1 element into allele encoding the cDNA 14d, or is an artifact of the cDNA library. The remaining new sequences (ΨS4, ΨS5, NS3, and ΨNS4) show considerable synonymous divergence (>5%, Tables 2 and 3) from any other (or each other) characterized gene, suggesting that each is a unique gene.

The three other genomic sequences (clones 205, 217, and 222) encode pseudogenes ΨNS4, ΨS4, and ΨS5 (Fig. 1 and Table 1). The ΨS4 and ΨS5 sequences are most similar to cow stomach lysozymes (Table 2, and see below), bringing the number of stomachlike genes to six (four expressed and two pseudogenes, Table 1). I have isolated by PCR part of exon 2 of two additional cow lysozyme genes (Fig. 2 and Table 1). The cow clones K2 and K3 are very similar to the cow kidney cDNA (Takeuchi et al. 1993; Steinhoff et al. 1994), and they predict amino acid sequences similar to the cow kidney lysozyme (Ito et al. 1993). These sequences (cow PCR clones K2 and K3, and kidney) probably represent the same gene. (See below.) A second type of amplified genomic sequence, cow NS3, is most similar to the pseudogene ΨNS4 and next most similar to the kidney lysozyme (Table 2). The N-terminal sequence of milk lysozyme and the amplified exon 2 sequence of NS3 overlap only for residues 35–39; therefore we cannot exclude the possibility that they represent the same gene. Therefore at a minimum there are four nonstomach genes: tracheal (clone 14d/208), kidney (PCR clones K2/K3), milk (NS3?), and a pseudogene ΨNS4 (clone 205).

As summarized in Table 1, genomic sequences derived from either genomic clones or PCR products identify ten lysozymelike genes (one S2 genomic sequence but two S2 genes). If the amplified exon 2 sequence cow NS3 is not the cow milk sequence then at least 11 genes must exist. At least seven genes are expressed (Table 1), six of which are represented by cDNA clones (Irwin and Wilson 1989; Takeuchi et al. 1993). The possibility exists that some of the sequences identified as alleles (e.g., of stomach genes or the trachea gene) or the amplified PCR products (cow K1–K4) actually represent additional genes. In another ruminant species, the goat, three tear lysozymes have been described (Jollès et al. 1990), one

Table 3. Percent synonymous (above diagonal) and nonsynonymous (below diagonal) divergence between lysozyme sequences[a]

	Cow S1	Cow S2	Cow S3	Sheep S1	Sheep S2	Sheep S3	Deer S1	Deer S2	Tra 208[b]	Tra 14d[b]	ψNS4	Kidney	Human	Rhesus	Mouse M	Rat 1
Cow S1	—	15.2	12.2	16.5	15.0	16.6	22.2	26.5	28.4	30.1	38.6	31.8	53.2	48.2	79.1	75.5
Cow S2	0.7	—	12.2	15.2	12.9	14.4	18.2	28.4	27.5	29.2	37.7	25.4	47.2	44.7	77.5	73.9
Cow S3	1.0	0.3	—	10.7	12.9	11.4	16.6	18.2	24.1	25.7	33.7	27.2	47.2	40.4	65.4	62.2
Sheep S1	1.3	1.3	1.7	—	8.5	7.2	18.1	21.4	30.4	32.1	36.8	31.0	50.7	42.6	72.8	72.4
Sheep S2	1.3	1.5	1.8	1.5	—	1.2	18.1	24.7	30.4	32.1	37.8	30.1	48.4	44.8	77.5	77.2
Sheep S3	1.7	1.8	2.2	2.5	1.0	—	16.5	23.0	28.6	30.3	35.8	28.2	46.2	42.6	74.3	74.0
Deer S1	2.9	3.4	3.7	2.7	3.0	4.1	—	15.7	37.5	39.4	47.0	39.4	60.0	56.0	81.4	81.1
Deer S2	1.0	1.7	2.0	1.7	1.7	2.7	2.2	—	35.6	37.5	54.4	42.8	68.7	56.2	81.7	88.6
Trachea 208	13.6	14.6	14.6	14.8	14.4	14.8	16.9	14.9	—	1.2	22.2	19.5	29.8	26.8	45.1	51.5
Trachea 14d	13.2	14.2	14.2	14.4	14.0	14.4	16.5	14.5	0.3	—	23.8	21.0	31.4	28.4	47.0	53.6
ψNS4	22.3	23.5	23.0	24.1	23.4	23.9	26.1	23.7	15.9	15.5	—	15.6	27.1	27.3	56.9	59.8
Kidney	18.6	19.8	19.3	20.1	19.5	19.1	21.8	19.8	17.0	16.6	14.4	—	21.3	23.3	59.6	64.4
Human	20.7	21.1	21.4	21.1	21.5	21.1	22.5	21.6	20.7	20.3	17.5	11.7	—	3.0	55.3	60.9
Rhesus	19.1	20.2	19.8	19.7	20.1	20.6	21.1	20.7	18.5	18.1	16.7	11.3	6.1	—	47.4	51.2
Mouse M	28.7	30.0	29.5	30.7	30.0	30.5	31.8	29.3	25.3	24.8	21.6	17.9	17.0	16.2	—	23.7
Rat 1	29.9	31.2	30.7	31.9	31.2	31.6	33.1	30.5	22.6	22.2	24.8	18.9	22.2	19.7	7.2	—

[a] Synonymous and nonsynonymous divergence estimated from coding region of mature lysozyme sequences, and corrected by the Jukes and Cantor method

[b] Tra refers to trachea

of which (tear 1) may be the homolog of cow milk lysozyme. While the cow does express lysozyme in tears, its identity is unknown (Prieur 1986). It appears that most, if not all, types of cow lysozyme genes have been identified and partially characterized.

Stomach Genes and Pseudogenes

Two genomic clones, cow 217 and cow 222, contained partial gene sequences ΨS4 and ΨS5, respectively (Fig. 1). Only a small portion of ΨS4 was found to be similar to other lysozyme genes: a segment that contains exon 2 and short regions flanking this exon, 91 bp 5′ and 27 bp 3′. The only other sequences within the 3,264 bases of sequenced DNA similar to any lysozyme gene sequence were pieces of repetitive DNA. Southern blot analysis showed that no other lysozymelike sequences were found within the two genomic clones (data not shown), which included at least 7 kb of both 5′ and 3′ flanking DNA. It appears that this lysozymelike gene, ΨS4, is composed of just this exon.

Only exons 1 and 2 were characterized for ΨS5, but these sequences were found at one end of a genomic clone; therefore it is possible that this gene may also contain exons 3 and 4. As with the cow stomach genes (see Irwin et al. 1993), the majority of the intron sequences of ΨS5 show considerably more divergence from the stomach genes (approximately 25%; data not shown) than do the silent sites of exons (about 13%, Table 2), or the intron sequences immediately adjacent to the introns. This pattern, low sequence identity of intron sequence distant from exons and higher identity at silent sites within exons, or in the immediately flanking intron sequences, is similar to that seen for the functional stomach genes (Irwin et al. 1993). It was concluded that the mosaic pattern of sequence similarity in the functional stomach lysozymes was due to the concerted evolution (Irwin et al. 1993). For both pseudogenes ΨS4 and ΨS5 the exon sequences show considerable sequence identity with the functional stomach lysozyme genes, but the introns show limited (ΨS5) or no (ΨS4) sequence identity, suggesting that these genes have also participated in the concerted evolution of the stomach lysozymes. Since the last concerted evolution event, these two genes have degraded into pseudogenes. Comparison of the numbers of synonymous and nonsynonymous substitutions per synonymous (d_S) or nonsynonymous (d_N) site respectively (data not shown), indicates that the ratio d_N:d_S is approximately equal to 1 on the lineages leading to ΨS4 and ΨS5, suggesting that these sequences have behaved like pseudogenes since their divergence from the functional stomach lysozyme genes. ΨS5 may have escaped concerted evolution when it moved away from the lysozyme gene cluster to its new chromosomal position. (See below.) The molecular events which resulted in ΨS4 becoming a pseudogene are less clear.

Nonstomach Lysozyme Genes

Multiple nonstomach lysozymes are known to exist in the ruminants, but unfortunately most are known only from partial N-terminal amino acid sequences (Jollès et al. 1990). The recent purification and sequencing of cow and sheep kidney lysozymes (Ito et al. 1993) and isolation of cDNAs for lysozymes expressed in the trachea, mammary glands, and neutrophils (Takeuchi et al. 1993;

Steinhoff et al. 1994) have provided a few complete sequences. As described in the Results, the genomic clone 208 appears to contain the expressed tracheal lysozyme gene. The genomic clone 205 was found to contain a lysozymelike pseudogene, ΨNS4, which is approximately equally different from stomach, tracheal, and kidney lysozyme sequences (Fig. 1, see Table 3). The aligned coding region of ΨNS4 shows that it possesses proline^{103}, an amino acid which has been deleted in the stomach lysozymes of advanced ruminants and in tracheal lysozyme (Fig. 1), and thus is like kidney lysozyme (Ito et al. 1993; Takeuchi et al. 1993; Steinhoff et al. 1994), camel stomach lysozyme, and the lysozymes of nonruminant species (Jollès et al. 1990). ΨNS4 shares lysine at position 2 with cow milk and goat tear lysozymes (Jollès et al. 1990) and may be more closely related to them than to any other lysozyme.

Multiple nonstomachlike lysozyme sequences were amplified from ruminant species (Fig. 2). The sequences appear to fall into two classes, with both classes present in each species. Sequences of cow clones K2 and K3 are nearly identical to the kidney cDNA and amino acid sequence (Takeuchi et al. 1993; Ito et al. 1993), with single base/amino acid differences at codon 34 of clone K2 and codon 63 of clone K3 (Fig. 2). The observed differences between cow clones K2/K3 and kidney lysozyme are probably mistakes introduced by PCR, and all of these sequences most likely represent the same gene—kidney lysozyme. The sheep sequence most similar to the cow kidney sequence, sheep clone K2, is consistent with the amino acid sequence of sheep kidney lysozyme (Ito et al. 1993). The goat (goat clones K1 and K3) and fallow deer (fallow clone K1) also have sequences similar to the sheep and cow kidney sequences (Fig. 2). The remaining ruminant sequences (cow NS3-like: cow clones K1/K4, sheep clones K1/K3, goat clone K2, and fallow clone K2) are very similar to each other and appear to represent the product of a gene that duplicated from the kidney lysozyme gene before the radiation of advanced ruminants (~25 million years ago; Savage and Russell 1983).

Cow Lysozyme Gene Cluster

Most of the bovine-lysozymelike genes have been found to map to band 23 of bovine chromosome 5, with one mapping to chromosome 7 (Gallagher et al. 1993). The genes located on chromosome 5 are all located within a 3.1-Mb *Mlu*I DNA fragment (Gallagher et al. 1993). As with the stomach lysozyme genomic clones (Irwin et al. 1993), none of the nonstomach lysozyme genomic clones studied here contained two genes. A 3.5-kb *Eco*RI fragment had been detected with lysozyme cDNA probes to be on chromosome 7, and had been suggested to be a pseudogene (Gallagher et al. 1993). Genomic clone cow 222, which encodes ΨS5, contains a 3.5-kb *Eco*RI fragment and probably represents this gene.

Phylogeny of Bovine Lysozyme Genes

Phylogenetic trees were constructed for the DNA sequences encoding the mature lysozymes using both the neighbor-joining method, from estimates of the synonymous and nonsynonymous divergences (shown in Tables 2 and 3), and by maximum parsimony (Fig. 3). The ruminant lysozyme DNA sequences were found to fall into three groups: (1) stomach lysozyme sequences, (2) tracheal lysozyme, and (3) kidneylike lysozymes. These same groups were observed if the partial sequences (ΨS4, ΨS5, NS3) were included in the analyses, or if only rodents or only primates were used as outgroups (data not shown). The stomach lysozymes were strongly clustered in all analyses (99–100% of bootstrap replications, Fig. 3). A closer association of tracheal lysozyme to the stomach lysozymes than to any other lysozyme sequence is moderately suggested by the trees (50–99% of bootstrap replications, Fig. 3). Tracheal lysozyme shares the unique deletion of amino acid 103 with the stomach lysozymes of the true ruminants. (See Fig. 1 and Takeuchi et al. 1993.) The third, paraphyletic, group consists of the kidney, ΨNS4, and NS3 lysozyme genes. These genes are most distantly related to the stomach lysozymes, and appear to represent two lineages (Fig. 3), the kidney lysozyme gene lineage and the NS3/ΨNS4 lineage. (NS3 and ΨNS4 cluster is supported by 97% of the bootstrap replications, Fig. 3C.) The relationship of these two lineages to the other genes are unclear. (Compare Fig. 3A to B and C.)

Phylogenetic trees of intron and 5′ flanking sequences were also constructed for the available complete gene sequences: the three cow stomach genes, and the new trachea and ΨNS4 gene sequences, with the human sequence used as the outgroup (Fig. 4). The portions of the gene sequence chosen for this analysis were those found not to evolve in a concerted fashion among the cow stomach genes (Irwin et al. 1993). As shown in Table 4, all of the cow sequences are nearly equidistant from each other, in contrast to the coding regions (Tables 2 and 3). The stomach genes still are each other's closest relatives, but are only slightly more related to each other than they are to either trachea or ΨNS4 (Fig. 4). The placement of the two remaining cow genes differs between the two trees (Fig. 4A and B). Parsimony analysis suggests that ΨNS4 is the outgroup to stomach and tracheal lysozymes, though neighbor-joining trees weakly (49% of bootstrap replications) support a closer relationship between ΨNS4 and the tracheal lysozyme (Fig. 4). The two alternative trees are not significantly different in their placements of the trachea and ΨNS4 genes. It seems clear that the various genes initially duplicated from each other over a short period of time.

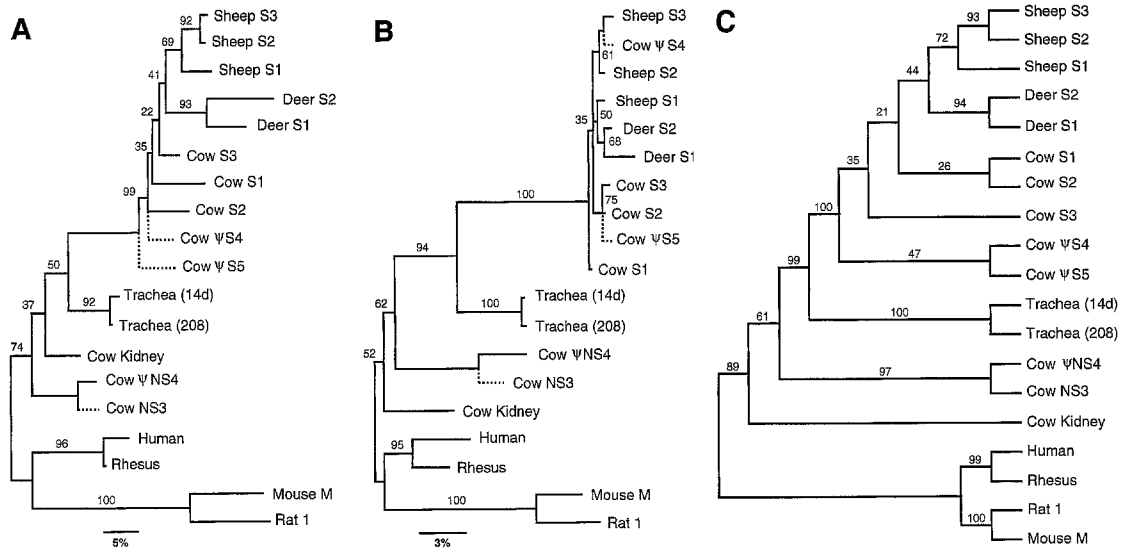

Fig. 3. Relationships of lysozyme coding sequences. The phylogeny of lysozyme and lysozymelike sequences (Figs. 1 and 2) was estimated by neighbor-joining of synonymous (**A**) or nonsynonymous (**B**) divergence, corrected by the Jukes and Cantor method, or by parsimony analysis (**C**) of all nucleotide differences among the coding sequences corresponding to the mature (i.e., secreted) lysozyme. Primate and rodent sequences were used to root the phylogenies. Partial sequences (ΨS4, ΨS5, and NS3) were excluded from the distance analysis; for illustration purposes their relative positions are indicated by the *dotted line* based on trees constructed from just the shared exon 2 sequences. Trees are the consensus of 500 bootstrap replications, with the neighbor-joining distances corrected by the Jukes and Cantor method. The percentage of bootstrap replications supporting each branch in the consensus tree is indicated above or to the right of the branch. Neighbor-joining trees (**A** and **B**) are shown with approximate branch lengths (except for partial sequences indicated by dotted lines), with the *bar* indicating either 5% (**A**) or 3% (**B**) divergence. The parsimony tree (**C**) indicates relationships only.

Comparison of phylogenetic trees derived from coding and untranslated portions of stomach lysozyme cDNAs indicated that the coding portion of the gene were evolving in concert, while the 3′ untranslated region was evolving divergently (Irwin and Wilson 1990). Characterization of gene sequences of the stomach lysozymes extended this observation and indicated that the concerted evolution events were limited to the coding exons—that is, the introns were evolving divergently (Irwin et al. 1993). Apart from the stomach pseudogenes, no obvious differences in divergence of intron and exon sequences were observed with the newly characterized lysozyme genes (Tables 3 and 4). In addition, trees derived from single exons, either coding or noncoding, yielded similar relationships between the stomach, trachea, kidney, and ΨNS4 lysozyme genes (results not shown). These results suggest that concerted evolution is not occurring between the nonstomach genes (trachea, kidney, and ΨNS4), or between the stomach and nonstomach genes.

Dates of the Early Gene Duplications

The analysis of genomic Southern blots had suggested that most of the duplications of the lysozyme gene in the ruminant lineage occurred after the divergence of the ruminants from the camel (~50 million years ago), with one duplication preceding this divergence but occurring after the pig–ruminant divergence (~55 million years ago) (Irwin et al. 1989, 1992). The dates of the early gene duplications, those leading to the kidney, ΨNS4, and tracheal genes, can be estimated from the silent divergence of coding sequences or from the divergence of introns among these genes. Comparison of several genes that have been sequenced in both cow and pig or cow and sheep suggested the divergence at silent sites within artiodactyls is approximately 0.7% per million years (0.35% per million years per lineage; Li et al. 1987; see also Irwin et al. 1993). As shown in Table 5, use of this assumption predicts that the time of divergence of the cow, sheep, and deer stomach genes from each other was 20–30 million years ago, an estimate in accord with divergence times of these species from other sources (Savage and Russell 1983; Irwin et al. 1991).

The cow tracheal gene is inferred to have diverged from the stomach genes approximately 45 million years ago, while the kidney and ΨNS4 genes are inferred to have diverged from the remaining genes 45–57 million years ago (Table 5). The calculated silent divergence of the cow nonstomach genes (tracheal, kidney, and ΨNS4), though, is lower than that estimated for the stomach genes (26.9% vs 46.8% with primates as the outgroup, or 54.7% vs 72.3% when rodents are the outgroup, Table 3). Since the stomach sequences appear to behave in a clocklike fashion within ruminants (see above), it appears that the divergence of the nonstomach sequences has been underestimated by the Jukes and Cantor correction method, or that the silent divergence

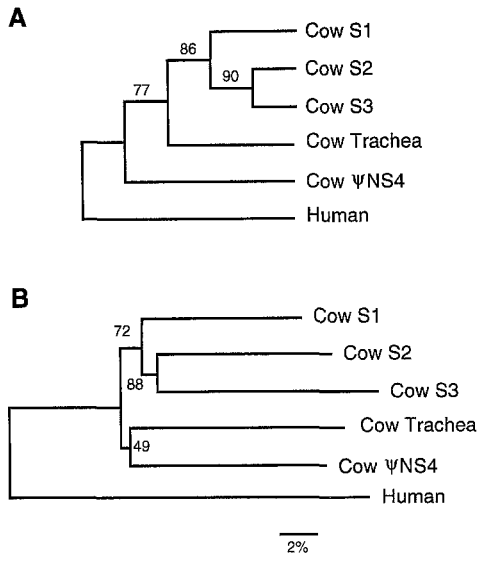

Fig. 4. Phylogeny of lysozyme intron and 5′ flanking sequences. The phylogenetic relationships of intron and 5′ flanking sequences that do not participate in concerted evolution among the stomach genes were assessed by parsimony **(A)** and neighbor-joining **(B)**. Trees are the consensus of 500 bootstrap replications, with the neighbor-joining distances corrected using the Kimura two-parameter method. The percentage of bootstrap replications supporting each branch in the consensus tree is indicated above or to the right of the branch. The neighbor-joining tree **(B)** is shown with approximate branch lengths, with the *bar* indicating 2% divergence. The parsimony tree **(A)** indicates relationships only.

Table 4. Percent divergence of 5′ flanking and intron sequences[a]

	Cow S2	Cow S3	Trachea	ψNS4	Human
Cow S1	19.6	21.7	22.9	22.7	35.5
Cow S2		21.9	23.7	23.3	39.0
Cow S3			25.9	25.3	42.8
Trachea				23.1	39.5
ψNS4					37.7

[a] Divergence estimated from 3,778 aligned bases of 5′ flanking and intron sequence which did not evolve in concert in stomach lysozymes. Distances were corrected by the Kimura two-parameter method

rate of nonstomach genes is lower than that for stomach genes. Therefore, it appears that the divergence time of the nonstomach genes from the stomach genes may be underestimated.

A second way that the divergence dates of the nonstomach genes from the stomach genes can be estimated is from the divergence of 5′ flanking and intron sequences. After excluding exons, and adjacent intron sequences, which may be evolving in concert (see Irwin et al. 1993), a total of 3,778 bases could be aligned among the five complete cow lysozyme sequences and the human gene sequence. In contrast to the situation at silent sites within the coding region, the estimated divergences of the stomach and nonstomach sequences are similar: All the cow genes show about 40% divergence from the human sequence (Table 4), yielding a divergence rate of approximately 0.5% per million year (if we assume cow and human diverged 75–80 million years ago). This estimate is lower than that estimated for silent DNA, approximately 0.7% per million years (Li et al. 1987), probably because some of the sequences are constrained. Using the divergence estimate of 0.5% per million year, one predicts that the cow stomach genes diverged from each other 39–44 million years ago, in accord with estimates based on the 3′ untranslated sequences of the cDNAs (Irwin and Wilson 1990). In addition, the nonstomach genes, trachea and ΨNS4, are estimated to have diverged from the stomach genes 45–52 million years ago, in agreement with the estimate from silent divergence (Table 5).

Table 5. Estimated dates of divergence of lysozyme genes

Genes compared	Silent divergence[a]	Date[b]
Stomach		
Cow vs sheep	14.0 (10.7–16.6)	19.9 (15.3–23.7)
Cow vs deer	21.7 (16.6–26.5)	31.0 (23.7–37.9)
Sheep vs deer	20.3 (16.5–24.7)	29.0 (23.6–35.3)
Stomach vs nonstomach		
Trachea vs stomach	31.2 (24.1–37.5)	44.5 (34.4–53.6)
Kidney vs stomach	32.0 (25.4–39.4)	45.7 (36.3–56.3)
ψNS4 vs stomach	40.2 (33.7–54.4)	57.4 (48.1–77.7)
Human vs ruminant		
Human vs stomach	52.7 (46.2–68.7)	75.3 (66.0–98.1)
Human vs nonstomach	27.4 (21.3–31.4)	39.2 (30.4–44.9)

[a] Average percent pairwise silent divergence, with range minimum to maximum estimates in parentheses, from Table 3

[b] Estimated date of divergence, in millions of years before present, for average divergence, with range of minimum to maximum in parentheses, and assuming a silent divergence rate of 0.7% per million years (see text)

Duplication of Ruminant Lysozyme Genes and the Divergence of the Camel

Estimation of divergence dates suggests that some of the nonstomach lysozyme genes, i.e., kidney and ΨNS4, diverged from the stomach genes before the divergence of camel and advanced ruminants (which occurred approximately 50 million years ago; Savage and Russell 1983), while others, i.e., trachea, diverged more recently. While the DNA sequence of either camel lysozyme gene is not available, the amino acid sequence of camel stomach lysozyme is (Jollès et al. 1990). Phylogenetic analysis of the ruminant lysozyme amino acid sequences yields similar relationships to those derived from the DNA sequences (Fig. 5). As with the analysis of DNA sequences (Figs. 3 and 4), the stomach sequences group together (99% of bootstraps), and the tracheal lysozymes are more closely related to stomach lysozymes than to other cow nonstomach lysozymes (ΨNS4, kidney) (60% of bootstrap replications, Fig. 5). As seen with the analysis

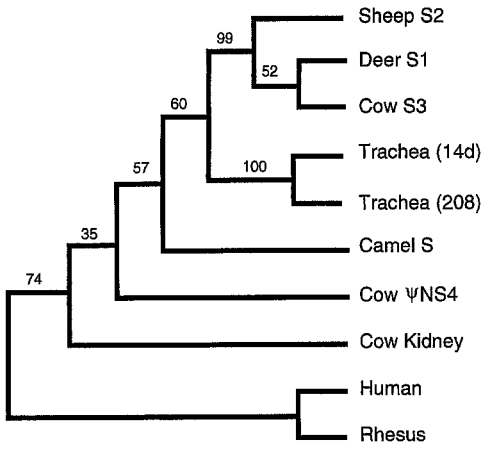

Fig. 5. Phylogeny of lysozyme amino acid sequences. Phylogenetic relationships of selected ruminant lysozyme sequences with primate lysozyme sequences used to root the tree. Phylogenetic relationship was assessed by parsimony, using the PROTPARS stepmatrix to determine number of steps for each amino acid change. Trees are the consensus of 500 bootstrap replications, with the percentage of replications supporting each branch indicated above each branch. If rodent lysozyme sequences were used to root this tree two differences in topology were observed: (1) the relationship of stomach lysozymes, with *deer* S1 clustered with *sheep* S2 (48% of bootstraps), and (2) kidney and ΨNS4 being clustered (29% of bootstraps). The remaining lineages were supported by a similar number of bootstrap replications.

of the DNA sequences, the kidney sequence and ΨNS4 were found not to be monophyletic (they were monophyletic if rodents were used as the outgroup, though this was supported by only 29% of the bootstrap replications; results not shown), and the kidney sequence is the most distantly related to the other ruminant lysozyme genes (Fig. 5).

The camel has two lysozyme genes (Irwin et al. 1989), and the product of one, stomach lysozyme, has been purified and sequenced (Jollès et al. 1990). It has been proposed that the first duplication of the ruminant lysozyme gene occurred prior to the divergence of the camel and advanced ruminants (Irwin et al. 1989, 1992). As expected, the camel stomach lysozyme falls within the ruminant lysozymes, but the tree presented in Fig. 5 suggests that two gene duplications (those leading to kidney and to ΨNS4 lysozymes), rather than one, occurred before the divergence of camel and advanced ruminants (Fig. 5). This conflict in number of gene duplications may be explained in two ways: Either kidney, ΨNS4, and NS3 (and other genes, e.g., tear and milk) form a monophyletic group, or one of the two lineages formed by the earliest gene duplications was lost. Characterization of the second camel lysozyme should resolve this question.

Ruminant Tracheal Lysozyme Evolved from Stomach Lysozyme

Surprisingly, the phylogenetic analysis of lysozyme amino acid sequences suggested that the stomach lysozymes of advanced ruminants were more closely related to cow tracheal lysozymes than to camel stomach lysozyme (Fig. 5). Phylogenetic trees constructed from the amino acid sequences of the various lysozymes from advanced ruminants (stomach, kidney, ΨNS4, and tracheal) and camel stomach lysozyme, with either primate or rodent sequences as an outgroup, show that as predicted, the stomach lysozyme genes of advanced ruminants (cow, sheep, deer) are more closely related to tracheal lysozyme than they are to camel lysozyme (Fig. 5). Closer examination of the sequences reveals that the ruminant tracheal and stomach lysozymes share deletion of amino acid 103 (Takeuchi et al. 1993; see Fig. 1), a deletion that is not found in camel stomach lysozyme (Jollès et al. 1990). Analysis of silent divergence estimates suggested that the tracheal lysozyme gene diverged from the stomach lysozyme genes of advanced ruminants approximately 45 million years ago (Table 5).

Since both the camel and advanced ruminants have a functional stomach lysozyme, i.e., a lysozyme that can function in the stomach (Dobson et al. 1984; Jollès et al. 1990), it is assumed that the ancestor of camel and advanced ruminants had a lysozyme which worked, to some extent, in the stomach. This would mean that the tracheal lysozyme has reverted, that is, has evolved from a lysozyme which could function in a stomach environment back to being a lysozyme that could function in a neutral environment. Alternatively, the most recent common ancestor of advanced ruminants and camel had a lysozyme like that of the pig, a stomach lysozyme which has not been adapted to function in the stomach environment. Consequently, we must then propose that since the divergence of the camel and the advanced ruminants that the stomach lysozymes have independently adapted to function in the stomach.

If the ancestor of camels and advanced ruminants did have a stomach lysozyme which could function in the stomach, we would expect this lysozyme to have some of the sequence properties of functional stomach lysozymes (Stewart et al. 1987). Tracheal lysozyme then would have evolved from this gene, and we may expect to find some of these adaptive changes retained in the modern tracheal sequence. Stomach lysozymes have fewer arginine residues than other lysozymes, presumably to reduce the number of potential pepsin cleavage sites (Stewart et al. 1987). Tracheal lysozyme has only four arginine residues (Fig. 2), a number similar to that of stomach lysozymes, and lower than ruminant nonstomach lysozymes such as cow kidney (eight arginines, Ito et al. 1993), or pig lysozyme (seven to nine arginines, Jollès et al. 1989). In addition, tracheal lysozyme shares with stomach lysozymes two amino acid residues previously identified as adaptive, lysine[21] and glutamic acid[126] (Stewart et al. 1987). Tracheal lysozyme also has a smaller number of acid-labile bonds (21) than nonstomach lysozymes (kidney has 27) or pig lysozyme (23–25), and similar in number to stomach lysozymes (cow S2 has

17). The above observations support the contention that the ancestor to camels and advanced ruminants had a functional stomach lysozyme (though it may have not been well adapted—see Irwin et al. 1992), and that ruminant tracheal lysozyme evolved from this lysozyme and reverted to function again in a neutral environment.

Evolution of Lysozyme Gene Expression

It was previously suggested that the first duplication of the lysozyme gene in the early ruminant allowed for the evolution of both a stomach and a nonstomach lysozyme (Irwin et al. 1989, 1992; Jollès et al. 1990). Stomach expression of lysozyme preceded duplication of the lysozyme gene, but adaptation to stomach function occurred only after duplication of the gene (Irwin et al. 1989, 1992). This appears to be another case of resolution of an adaptive conflict (Wistow 1993). That is, in the ancestor to ruminants, the single lysozyme gene could not carry out both stomach and nonstomach functions, but duplication of the gene allows the two genes to carry out both roles. (Leaf-eating monkeys appear to have resolved this conflict without gene duplication [Stewart et al. 1987; Swanson et al. 1991].) The suggestion that tracheal lysozyme is derived from a stomach lysozyme shows that the nonstomach lysozymes of ruminants are not a monophyletic group of genes. A second change in lysozyme gene regulation is required. At least one of the stomach lysozyme genes is expressed in tracheal cells (Takeuchi et al. 1993), and this therefore suggests that ancestral stomach lysozyme may have been expressed in both the stomach and the trachea. A second case of adaptive conflict may have occurred, and subsequent duplication of the lysozyme gene could have released this conflict and allowed the evolution of modern tracheal lysozyme. The tracheal lysozyme gene was then free to revert to function in the neutral environment of the trachea.

The ruminant lysozyme gene family continues to illustrate unexpected aspects of the evolution of a gene family. Within the ruminant lysozyme genes concerted evolution is limited to only the stomach genes (both functional and pseudogenes), though preliminary evidence had suggested it may have also occurred between the nonstomach genes (Jollès et al. 1990). Change in lysozyme gene expression and lysozyme function has occurred several times within the ruminant lineage. A single origin of stomach and nonstomach expression patterns and a unique adaptation of lysozyme function are unlikely to be due to the close relationship of tracheal and stomach lysozymes. These results emphasize the potential of multiple changes in the regulation and function of genes within a gene family.

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