Multiple cDNA Sequences of Bovine Tracheal Lysozyme*

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The principal role of lysozyme is to prevent bacterial invasion at body surfaces. We are interested in how lysozyme is regulated at the surface of the respiratory tract, where the serous gland cell is regarded as the primary cellular source of this enzyme. Since the cow genome contains at least 10 lysozyme-like genes, our objective was to determine which of them are expressed in the cow tracheal cDNA library. For screening tracheal cDNA libraries with a probe constructed from the cDNA encoding stomach lysozyme 2, we obtained 3 lysozyme cDNAs: 5a (1023 base pairs (bp)), 7a (1060 bp), and 14d (1249 bp). cDNA 7a corresponds to a previously reported gene (showing sequence identity to the stomach 2 lysozyme gene), whereas cDNAs 5a and 14d correspond to lysozyme genes not previously reported. Northern blot analysis of cow tracheal RNA showed lysozyme mRNAs of three distinct lengths. Based on hybridization with probes specific for each cDNA, we determined that the longest transcript corresponded to cDNA 5a, the shortest to 7a, and the intermediate-length transcript to 14d. Cultured cow tracheal gland serous cell RNA, reverse transcribed and amplified by the polymerase chain reaction with primers common to all three cDNAs, we obtained the cell walls of most bacteria. Although lysozyme's principal role is in host defense (Masson, 1973), foregut fermenting mammals (ruminants and leaf-eating monkeys) use lysozyme to lyse bacteria and expose their contents to mammalian digestive enzymes (Dobson et al., 1984). The multiple-functional role of lysozyme in these species has been made possible through the preservation of adaptive mutations associated with gene duplication (for review, see Irwin et al. (1992a)).

Unlike the human genome, which contains a single lysozyme gene (Castanon et al., 1988; Peters et al., 1989), the genomes of many other species contain two or more (Irwin et al., 1992b). The cow genome contains at least 10 lysozyme genes, four of which are expressed in the stomach (Irwin et al., 1989). Other potential sites of lysozyme expression in the cow are mammary, salivary, lacrimal, and tracheobronchial tissue, as well as neutrophilic, eosinophilic, and monocytic leukocytes (Klockars and Osserman, 1974; Klockars and Raitano, 1975; Mason and Taylor, 1975).

In human tracheobronchial tissue, lysozyme has been detected in submucosal gland serous cells (Bowes and Corrin, 1977; Spicer et al., 1977) as well as in the surface epithelium (Konstan et al., 1982). Lysozyme is subject to developmental regulation at these tissue sites, as shown by approximately 2-fold increases in the lysozyme concentration found in bronchial explant medium from premature (26–32 weeks of gestation) versus mature (40 weeks of gestation) newborns (Boat et al., 1977). In view of lysozyme's important role in host defense, it may also be regulated in tracheobronchial cells by bacterial by-products or inflammatory mediators (e.g. TNFα, lipopolysaccharide, and γ interferon), as has been demonstrated in cultured human monocytes (Lewis and McGee, 1990). To address the issue of tracheal lysozyme regulation in a model system, we have developed cultures of serous gland cells from the cow trachea (Finkbeiner et al., 1986). Identification of the lysozyme gene(s) expressed by these cells is a necessary first step in the investigation of lysozyme gene regulation. To this end, we have examined the repertoire of lysozyme isoforms and mRNAs expressed in the cow trachea as a whole and then obtained evidence indicating that one particular cDNA (5a) encodes an isoform that is the principal form of lysozyme synthesized in the cow tracheal gland serous cell.

EXPERIMENTAL PROCEDURES

Tissue Extraction and Lysozyme Overlay Gels—Fresh bovine tracheas and stomach tissue were transported from the slaughter house on ice and dissected within 2 h post mortem. After dissection, the various types of sample (whole trachea, microdissected epithelium, microdissected glands, stomach tissue) were finely chopped and homogenized in two volumes of 2% acetic acid, 2% 2-phenoxyethanol and then left at 4 °C overnight. Extracts were spun at 27,000 × g for 20 min, and supernatants were saved for assay. Nondenaturing gel
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7a

GCTGAGCATAGGAGCTGAGCGGAAACCCGAGATTTAG

KT 20

--- A-C-G-G---

14D

AGGAGCTGCTATTCTGTGAGACCTGTC

5A

AGGAGCTGCTATTCTGTGAGACCTGTC

KT 27

--- A-C-G-G---

14D

AGGAGCTGCTATTCTGTGAGACCTGTC

5A

AGGAGCTGCTATTCTGTGAGACCTGTC

KT 17

--- A-C-G-G---

14D

AGGAGCTGCTATTCTGTGAGACCTGTC

5A

AGGAGCTGCTATTCTGTGAGACCTGTC

KT 28

--- A-C-G-G---

14D

AGGAGCTGCTATTCTGTGAGACCTGTC

5A

AGGAGCTGCTATTCTGTGAGACCTGTC

Fig. 1. Schematic indicating the positions of primers used for RT-PCR, primer extension analysis, and cDNA-specific probes for Northern blots.

| Table I |
| List of primers |
| --- | --- | --- | --- |
| Name | Sequence (from 5’ to 3’) | Position | Orientation | Specificity |
| DI4 | AGGGGTTTGGAGGATG | 172-188 | Sense | None |
| D19 | AGGGGTTTGGAGGATG | 228-245 | Antisense | 7a, 14d |
| D11 | AGGGGTTTGGAGGATG | 366-384 | Antisense | None |
| KT14 | AGGGGTTTGGAGGATG | 496-513 | Antisense | None |
| KT17 | AGGGGTTTGGAGGATG | 210-238 | Antisense | 5a |
| KT20 | AGGGGTTTGGAGGATG | 106-127 | Sense | None |
| KT21 | AGGGGTTTGGAGGATG | 322-343 | Antisense | None |
| KT27 | AGGGGTTTGGAGGATG | 175-192 | Antisense | 14d |
| KT28 | AGGGGTTTGGAGGATG | 306-323 | Antisense | 7a |

electrophoresis was performed at pH 4.3 in 10% polyacrylamide, and the gels were overlaid with a “substrate gel” consisting of 7% acrylamide (at pH 5.0 or 7.0) containing 1 mg/ml Micrococcus lysodeikticus cells (Hammer et al., 1987). After a 30-60-min incubation at 37°C, the substrate gel was removed and incubated an additional 2-4 h at 37°C until bands of cell lysis appeared. The substrate gel was then placed in 0.1% SDS to inhibit the lysozyme activity until photographed.

Screening Bovine Tracheal cDNA Libraries—Aliquots of bovine tracheal cDNA prepared by oligo dT or random priming of cow tracheal epithelial mRNA were generously provided by Dr. E. Sigal (Cardiovascular Research Institute, University of California, San Francisco). The cDNA was packaged into the EcoRI site of XZAP I1 (Stratagene) using Gigapack Gold (Stratagene) as a packaging kit. A total of about 300,000 phage clones from one oligo(dT)-primed and one random primed library were plated, and plaque lifts on replicate nitrocellulose filters were made. For screening, the filters were hybridized with probes constructed from the following: a 213-bp, 7a-specific PCR product generated using primers DI 4 and DI 11; a 230-bp HINCl restriction fragment of the 5a cDNA and a 278-bp HINCl restriction fragment of the 14d cDNA. Hybridization and washing were performed under high stringency conditions. The blot was exposed for 5 days (for tracheal samples) and 2 h (for stomach sample).

PCR of RNA from Cultured Bovine Gland Serous Cell.—RNA was extracted from cultured bovine tracheal gland serous cells (Finkbeiner et al., 1986). The RNA was reverse transcribed with a lysozyme-specific primer, KT 14. Two additional primers (KT 20 and KT 21) for PCR were made from a highly conserved portion of the lysozyme coding region, identical to all three cDNAs. Each primer was based on cDNA sequence, but the sequence was altered to include an EcoRI

The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; UT, untranslated; kb, kilobase(s); RT, reverse transcriptase.
RESULTS

Analysis of Lysozyme by Electrophoresis and Substrate Overlay Gels—Tracheal tissue extracts contained lysozyme isoforms showing varying mobility on nondenaturing gels (Fig. 2). Bands A, B, and D were present in tracheal but not stomach extracts, whereas band C was present in both. Band C corresponded to the migration position of cow stomach lysozyme. Lysozyme from microdissected glands (Fig. 2, lanes 1 and 4) presented a band with mobility intermediate between that of cow stomach lysozyme and the fastest moving tracheal isozyme.

Sequence of Cow Tracheal Lysozymes—cDNA 7a was isolated from an oligo(dT)-primed library, and cDNAs 5a and 14d were isolated from a random primed library. Fig. 3 shows that the three cDNAs are approximately 85% identical in DNA sequence within the coding region. The 3′ halves of the 3′-UT regions were dissimilar, suggesting that each cDNA encodes a portion of a different gene. In the 3′-UT region of cDNA 5a, two Nla elements (Li et al., 1991; Spence et al., 1985) were present (Fig. 3). Both 7a and 14d have longer 5′-UT regions than those of lysozymes reported to date. The sequence of 7a closely resembled the four cow stomach 2 cDNAs previously described (cow 2a-d) (Irwin and Wilson, 1989). Clone 7a differs, however, from all four of these cDNA sequences at nucleotide 1050, containing a G in contrast to a C corresponding to the migration position of cow stomach 2 lysozyme (Fig. 4), possibly denoting an additional upstream exon not previously described. The 3′-UT region of 7a contained a polyadenylation signal and poly(A′) tail identical to those already described for stomach 2 lysozyme (Irwin and Wilson, 1989). The 3′-UT region of 14d had two polyadenylation signals (5′-AATAAA and AATATA) both upstream of poly(A′) tails. We found no polyadenylation signal or poly(A′) tail in cDNA 5a.

Predicted Amino Acid Sequences and Isoelectric Points—In each of the three cDNAs, we found an initiation codon (positions 118–120, Fig. 3) and a termination codon (positions 562–4, Fig. 3). Based on these, the predicted mature amino acid sequence is 120 residues long for 5a and 129 residues long for 7a and 14d (Fig. 5). Each mature lysozyme protein is preceded by a predicted signal peptide. The amino acid sequence predicted from cDNA 7a is identical to that reported for stomach lysozyme 2 (Irwin and Wilson, 1989). Calculated isoelectric points are 9.12, 6.49, and 10.00 for 5a, 7a, and 14d, respectively.

Primer Extension Analysis—Primer extension performed on tracheal RNA using a 5a-specific primer (KT 17) revealed a short 5′-UT region (28 bp, data not shown) in transcripts of this gene. The length of the 5a 5′-UT region is consistent with that of all four lysozyme genes expressed in the stomach, which also occur at approximately 28 nucleotides upstream of translation initiation (Fig. 6, lane 1, arrow A), and similar to the lengths of 5′-UT regions of lysozyme genes from other mammals (Cross et al., 1988; Peters et al., 1989; Swanson et al., 1991). Primer extension using a primer common to both cDNAs 7a and 14d (DI 9) revealed the existence of transcription start sites at about 230 (Fig. 6, arrow B) and 400 (Fig. 6, arrow C) nucleotides upstream of the translation start site in the tracheal glands (Fig. 6, lane 2) and epithelium (Fig. 6, lane 3). Because the 18-base oligonucleotide primer used for this experiment (DI 9) has three nucleotide differences from the corresponding 5a sequence, the primer should not anneal to 5a RNA under our conditions but could anneal to both 7a and 14d RNA. Although we have not been able to individually correlate 7a and 14d with the −230- and −400-bp start sites, it is clear that the trachea synthesizes lysozyme transcripts with considerably longer 5′-UT regions than does the stomach.

Whereas the relative intensities of the −230 and −400 bands are approximately equal in the epithelium (Fig. 6, lane 3), the −230 band is stronger than the −400 band in the glands (Fig. 6, lane 2), indicating that 7a and 14d mRNAs are expressed in different amounts. A subsequently performed experiment (see Fig. 8) showed that mRNAs corresponding to all three cDNAs are synthesized in gland cells, in the following relative abundance: 5a>14d>7a.

Northern Blot Analysis of Stomach and Tracheal mRNA—While there is little variation in the size of cow stomach lysozyme mRNA (Irwin and Wilson, 1989), transcripts of three sizes (ranging between 1.0 and 1.7 kb) are present in the trachea (Fig. 7). Based on hybridization with probes specific for each cDNA, we determined that the longest transcript corresponded to cDNA 5a, the shortest to 7a, and the intermediates-length transcript to 14d.

PCR and Southern Blot Analysis of RNA from Cultured Bovine Gland Serous Cells—RNA from cultures of bovine tracheal gland serous cells was analyzed by reverse transcriptase (RT)-PCR and Southern blotting with 5a-, 14d-, and 7a-specific probes. Primers used for amplification were from a conserved portion of the 5′-UT and coding regions and would amplify mRNA corresponding to any of the three cDNAs. The RT-PCR product corresponded to an ethidium bromide-stained band of the expected size, 238 bp. Southern blotting and hybridization (Fig. 8) showed that this band hybridized most strongly to a 5a-specific probe and less strongly to 7a-
FIG. 3. Sequences of three cow tracheal lysozyme cDNAs. The complete nucleotide sequence of cDNA 7a is shown, with substitutions in the other sequences indicated below. Asterisks denote gaps (insertions or deletions) required for sequence alignment. Two Ns repetitive elements in the 3' half of the 3'-UT region of clone 5a are underlined.

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FIG. 4. Comparison of 5'-UT region of clone 7a with stomach 2 genomic sequence. The 5' end of the clone 7a contained 89 bp not present in a full-length stomach 2 cDNA but present in the stomach 2 gene. The sequence includes a TATA box (underlined). The 11 bp at the extreme 5' end of cDNA 7a (also underlined) differs from the contiguous sequence of the stomach 2 gene.
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7a KYFVERCELAR TLKKGLDGKY KGVSANWLC LTKWESSYNT KNATNYPSSSE SDYIFQIN SKWNCNDDKT
14d -T-K----- -N---A-- ----------M- -A-G--N--- Q-K-----G-K -------------
5a ------------ S--RF-M-NF R-I-----M- -AR--N-- Q-----AGDQ ------------- -H-----

Fig. 5. Amino acid sequences of mature cow tracheal lysozymes. The predicted amino acid sequences of tracheal lysozymes from cDNAs in Fig. 3 are aligned. The clone 7a sequence appears above, with differences found in other sequences shown below. Asterisks denote deletions relative to other mammalian lysozymes (Dobson et al., 1984).

7a PNAVGDCHVS CSELMDIA KAVACAKHV SE*QITAWV AWKSHCRDHD VSSYVEGCTL
14d -K--N--G-- -- A-LKD--T Q------K-- -Q*------ ----N--K--NR-- LT--K--GV
5a -G--NA--LP -GA-LQD--T Q--------RV- -DP---R-- ----R--QNQ- LT--IQ--GV

Fig. 6. Identification of mRNA start sites. The DI 9 primer-extended products of tracheal mRNA (lanes 2 and 3) are shown together with that of stomach mRNA (lane 1). 5 μg of poly(A)+ RNA was used for trachea, and 1 mg of total RNA was used for stomach. Arrow A indicates the transcription start site for stomach RNA, and arrows B and C indicate those for tracheal RNA.

and 14d- specific probes. This indicates that the 5a-cognate gene is the major lysozyme gene expressed in cow tracheal gland serous cells.

DISCUSSION

Tracheal Lysozyme cDNAs—The lysozymes expressed in the cow trachea are encoded by at least three distinct lysozyme cDNAs (Figs. 3 and 5). That these correspond to distinct genes is indicated by the existence of major dissimilarities in the 3'UT regions of the cDNAs and the presence of a significant number of substitutions in the coding regions

resulting in different amino acid sequences). The mRNAs transcribed from these genes range in size between 1.0 and 1.7 kb (Fig. 7). These mRNAs direct the translation of iso-zymes showing a variety of mobilities on nondenaturing gels (Fig. 2). Since the three predicted proteins are similar in size, we assume that the primary determinant of their mobility is charge, reflected by isoelectric point. We also assume that tracheal band C (Fig. 2), which comigrates with stomach lysozyme, corresponds to cDNA 7a, because it encodes a protein identical to stomach 2 lysozyme. Having higher iso-electric points than 7a, cDNAs 5a and 14d likely encode the more rapidly moving proteins (based on predicted isoelectric point, 5a putatively corresponds to band B and 14d to band A in Fig. 2). The correspondence of 5a to band B is supported by data showing that 1) the principal band in concentrated extracts of microdissected glands (Fig. 2, lane 1) is band B, and 2) the 5a gene product is the principal lysozyme expressed by cultured gland serous cells (Fig. 8). Despite exhaustive screening of two libraries, we did not find a cDNA encoding a protein with a predicted isoelectric point low enough to correspond to band D. This leaves open the possibility that a fourth lysozyme gene is also expressed in the cow trachea.

Tracheal Isozymes Appear to Vary Widely in Net Charge and Arginine:Lysine Ratios—An unexpected finding was the presence of three isozymes with strikingly diverse electrophoretic mobilities. In the cow stomach, in which at least four separate genes are expressed (Irwin et al., 1989), the electrophoretic mobility of the gene products is relatively similar (Fig. 2), consistent with their similar isoelectric points. The distinct charges carried by the tracheal isozymes may contrib-
The significance of the elevated arginine:lysine ratio in the of individual isoforms to perform bacteriolysis (Fleming, the predominant form being 5a (Fig. 8). The more sensitive methods of PCR amplification and Southern blot analysis (Fig. 8), however, indicated that gland cells in fact synthesize mRNA corresponding to all three cDNAs, the predominant form being 5a (Fig. 8).

Cellular Origin of the Tracheal Isozymes—Substrate gel electrophoresis of protein from microdissected glands and epithelium (Fig. 2) suggested that the fastest and slowest moving isozymes are products of the epithelium, whereas isozymes of intermediate mobility are products of the glands. The more sensitive methods of PCR amplification and Southern blot analysis (Fig. 8), however, indicated that gland cells in fact synthesize mRNA corresponding to all three cDNAs, the predominant form being 5a (Fig. 8).

The Potential Contribution of Leukocytes to Tracheal Lysozyme—Since macrophages, neutrophils, and eosinophils are known to synthesize lysozyme and are present in variable numbers in tracheal tissue, it is possible that mRNAs from these cell types were present as “contaminants” during the construction of the cDNA libraries we screened. If so, cDNAs corresponding to leukocytic mRNA could have been isolated during our screening procedure. Although we cannot rule this out, it is clear that independent of the original source of the cDNA, mRNA corresponding to all three cDNAs (5a, 7a, and 14d) is present in cultured serous gland cells (Fig. 8).

Tracheal Lysozyme Transcript Lengths Assessed by Northern Blot—Inspection of the length of lysozyme mRNA transcripts by Northern blot revealed interesting differences between the trachea and the stomach (Fig. 7). Whereas stomach RNA essentially contained lysozyme transcripts of 1 kb, tracheal RNA contained transcripts of three distinct lengths, ranging between 1 and 1.7 kb. These differences cannot be explained by variations in the lengths of the coding regions (which are almost identical for all three cDNAs), and thus derive from differences in the lengths of the 5'- and 3'-UT regions. The use of probes specific for each of the cDNAs permitted us to define the length of the mRNA corresponding to each cDNA: 5a = 1.7 kb; 7a = 1.0 kb; and 14d = 1.3 kb.

Tracheal-specific Processing of the 7a/Stomach 2 Lysozyme Gene—It was previously thought that lysozyme genes expressed in the stomach were not expressed in other tissues (Dobson et al., 1984), since the amino acid sequence of cow stomach lysozyme has unusual features that were believed to reflect adaptation to stomach conditions (Dobson et al., 1984; Jolles et al., 1984; Stewart and Wilson, 1987). For example, cow stomach lysozyme has lost an acidic aspartyl-prolyl bond (Juaregui-Adell and Marti, 1975) between residues 102 and 103 and also has fewer amidases than other lysozymes (Jolles et al., 1984). The significance of the expression of the 7a/stomach 2 lysozyme in the respiratory mucosa, where pH is essentially neutral (Kyle et al., 1990) and protease activity is normally low, is unclear at this point. It is possible, however, that the relatively low net positive charge carried by this isozyme is important in keeping it dissociated from polyanionic mucins and that its resistance to proteolysis protects against proteases (Döring et al., 1983; Buttle et al., 1990) released during airway inflammation.

Tracheal Lysozyme mRNAs Have Longer 5'-UT Regions than Other Lysozymes—Primer extension analysis using a primer common to both 7a and 14d cDNAs revealed transcription start sites at approximately 230 and 400 bp upstream of the translation initiation codon. Thus, the 7a/stomach 2 lysozyme gene uses a transcription start site in the trachea at least 230 bp upstream of the AUG, in marked contrast to the start site used in the cow stomach (Fig. 5), langur stomach (Swanson et al., 1991), and mouse macrophage (Cross et al., 1988) (at 28 bp upstream). The tracheal mRNA may be formed by one of two mechanisms. First, in the trachea, RNA polymerase may use a second start site upstream of the stomach start site in Exon 1. Alternatively, 7a may span an intron, with its transcription start site occurring in a previously unrecognized upstream exon. A putative TATA box present in cDNA 7a, approximately 25 bp upstream of the transcription start site of stomach 2 (within the 5'-UT region of 7a), may represent part of the promoter that drives transcription of this lysozyme gene in the stomach; an alternate promoter, as yet unidentified, would presumably drive transcription of this gene in the trachea. The tissue-specific use of alternate promoters has been described for other genes (Hanke and Storti, 1988; Izzo et al., 1988; Samuelson et al., 1990).

Amino Acid Sequences Predicted from cDNAs: Evolutionary Considerations—The differences between the predicted amino acid sequences of mature cow tracheal lysozymes (14d, 5a, and 7a) are 32–44 residues. This is slightly fewer than the differences between cow stomach and pig lysozymes (44–53 residues) (Jolles et al., 1989) and approximately the same as the differences between camel stomach and cow-goat-deer stomach lysozymes (36–38) (Jolles et al., 1990). This suggests two possibilities as follows: 1) the genes encoding these three lysozymes diverged from each other at approximately the same time as the divergence of camel and cow (approximately
50 million years ago), or 2) there has been accelerated evolution of the protein sequences, perhaps due to acquisition of new, tissue-specific functions. The 14d sequence contains a deletion of residue 104 as do the stomach lysozymes of cow, sheep, goat, and deer, while most mammalian lysozymes, including pig and camel stomach, as well as 5a, do not have this deletion. This may signify that the amino acid deletion occurred after the camel-cow divergence, and that 14d is more closely related to cow (deer, etc.) stomach lysozymes than to camel stomach or 5a. The characterization of additional genes in key species will be required, however, to fully resolve the dates of these duplications.

In summary, the cDNAs we have isolated from the cow trachea apparently correspond to three distinct genes. These seem to have arisen through duplication and divergence from an ancestral lysozyme gene in the artiodactyls. The major isoform expressed by cow serous gland cells is encoded by the 5a gene in the airway gland serous cell. The characterization of additional genes in key species will be required, however, to fully resolve the dates of these duplications.

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