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Exons encode functional and structural units of chicken lysozyme

(mRNA sequence/introns/gene structure/evolution)

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ABSTRACT **The nucleotide sequence was determined for the chicken egg white lysozyme mRNA and for the exons of the gene together with their flanking intron regions. The exon pattern is to some degree related to the structural subdivision of the final protein product. However, the relationship of exons to functional units of the enzyme is better established. Exon 2 codes for amino acids 28–82, which include the catalytically active residues and a cluster of amino acids which bind rings C, D, E, and F of the oligosaccharide substrate. Exon 3 codes for amino acids 82–108, which give additional substrate specificity, determine the cleavage frame for the alternating *N*-acetylglucosamine/*N*-acetylmuramic acid chain, and increase the catalytic efficiency of the active center. Exons 1 and 4, respectively, code for translational signal sequences on the mRNA, for the signal peptide of prelysozyme, and for the amino- and carboxy-terminal regions of the enzyme. These regions increase the stability of the molecule but are not directly involved in the catalytic function.**

The discovery of the exon/intron structure of eukaryotic genes, requiring RNA splicing for the generation of mRNA, has led to new ideas about evolution of genes. It was suggested that the formation of new genes could be greatly facilitated by recombinational events within intervening sequences (1–6). Much of the strength of this hypothesis depends on the outcome of the question whether exons encode integral parts of proteins (6–8). Work in which the exon/intron structure of immunoglobulin genes was compared to structural and functional elements of their final gene product gave a surprisingly clear answer (9). Each of the functional domains of the mouse γ_1 and α heavy chain is encoded on a separate exon in the gene (9, 10). Inspection of hemoglobins in respect to the structure of their genes yielded more support for the idea that exons correspond to functional units of proteins. The central exon of the globin gene contains all the information for the heme-binding domain of the protein (6, 11). The distribution of globin intersubunit contacts among the three coding sequences shows a strong correlation as well (12).

The gene for chicken lysozyme was previously isolated and preliminarily characterized (13–16). Here we report its exact exon/intron structure as determined by DNA sequence determination. The result opens the possibility of discussing domain–exon correlation for one of the structurally and functionally best known enzyme proteins.

MATERIALS AND METHODS

Lysozyme Specific DNAs. DNA was prepared from λ Charon phages containing chicken lysozyme gene specific DNA, λlys30 and λlys31 (14), and the lysozyme mRNA specific cDNA plasmid pls-1 (17) as described. Isolated *Hin*dIII fragments were recloned in pBR322 and pUR51 (18) and in M13mp5 (19).

Chain Termination Sequence Determination. For sequence determination with chain-terminating inhibitors (20) we used as templates single-stranded M13mp5 DNA (21) containing lysozyme gene fragments. Primers were double-stranded restriction subfragments. Primers of more than 60 base pairs were pretreated with exonuclease (21). The same primers could be used on templates with inserts in opposite orientation. For chain termination sequence determination on partially purified lysozyme mRNA we used the method described by Levy *et al.* (22).

Nick-Translation Sequence Determination. 5′-End-labeled DNA fragments (23) were incubated with DNA polymerase containing DNase (Boehringer) according to the procedure of Seif *et al.* (24) in the chain-terminating "forward" and "backward" reaction.

Biosafety Conditions. Construction and growth of recombinant plasmids and phages were conducted under L2/B1 conditions as specified by the Zentrale Kommission für Biologische Sicherheit of the Federal Republic of Germany.

RESULTS

Primary Structure of the mRNA for Chicken Egg White Lysozyme. We have previously shown by Southern hybridization of total cellular DNA (13) and by Southern hybridization and electron microscopic analysis of hybrids of lysozyme mRNA with cloned DNA (14, 15) that the lysozyme gene contains several intervening sequences. The final decision about the number, size, and location of exons, however, can only be made by comparing the nucleotide sequences of the gene and its respective mRNA. In order to define the exon/intron junctions, we determined the complete sequence of lysozyme mRNA. The major part of the sequence was derived from the lysozyme cDNA plasmid pls-1 (Fig. 1*A*) constructed previously in our laboratory (17). The sequence of the 5′ noncoding region and the codons for the first 11 amino acids of prelysozyme was determined by priming cDNA synthesis on lysozyme mRNA with a DNA fragment from pls-1 in the presence of dideoxynucleotides (22). Modified S1 nuclease mapping (25) was used to define independently the start of the mRNA sequence. According to this result (unpublished data) the identity of the most likely 5′-terminal nucleotides was taken from the corresponding sequence of the cloned gene DNA.

Fig. 2 shows the complete structural gene sequence for chicken egg white lysozyme. The mRNA has a relatively short 5′ noncoding region of 29 nucleotides with some bases complementary to the 3′ end of 18S RNA (26). The presumptive ribosomal binding site could include 5 G·C base pairs. The UGA (opal) translational stop signal is followed by a 3′ noncoding region of 113 nucleotides. Unlike the case in other genes (i.e., refs. 27–29) the exact poly(A) addition site can be determined

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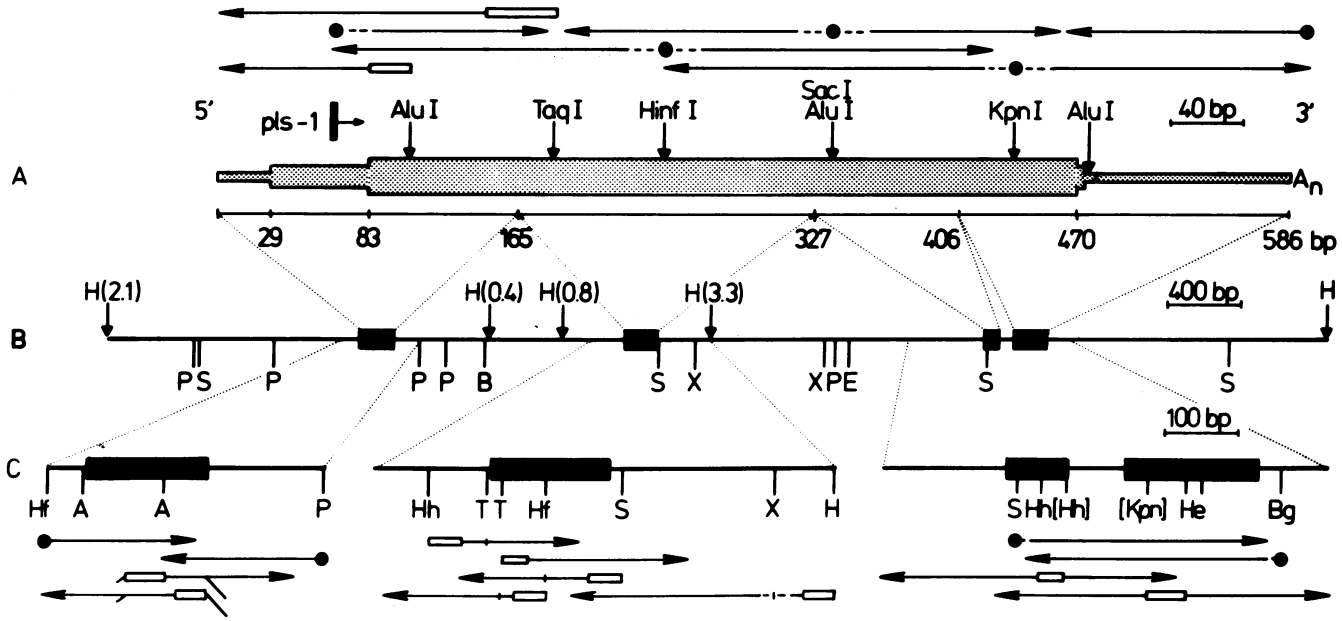

FIG. 1. Map of the chicken lysozyme gene. (*A*) Schematic representation of lysozyme mRNA with increasing widths of the bar representing noncoding regions and prelysozyme and lysozyme structural gene parts. The 5′ end of the cDNA clone pls-1 (17) within the sequence coding for the prelysozyme signal peptide is indicated. (*B*) Map of the lysozyme gene as derived from Southern hybridization, electron microscopic analysis (14, 15), and DNA sequence. (*C*) Enlarged exon sections are shown with flanking regions. Restriction enzyme cleavage sites for *Alu* I (A), *Bam*HI (B), *Bgl* I (Bg), *Eco*RI (E), *Hin*dIII (H), *Hin*fI (Hf), *Hha* I (Hh), *Hae* III (He), *Kpn* I (Kpn), *Pst* I (P), *Sac* I (S), *Taq* I (T), and *Xba* I (X) are indicated in the respective sections. Bracketed sites are restricted to the cDNA clone. DNA sequencing strategy is pointed out in *A* and *C*, where dots represent the labeled 5′ ends of fragments sequenced with the nick-translation method and open bars indicate the 5′ section of the primers used for sequencing with the chain-terminating method.

here because C at position 586 in the gene is not followed by an A but by a T. The conserved A-A-U-A-A-A sequence normally found in eukaryotic mRNAs around 20 bases before the poly(A) addition site (30) is slightly altered into the sequence A-U-U-A-A-A-A.

The coding sequence of 441 nucleotides (147 codons) includes

FIG. 2. DNA sequence corresponding to chicken egg white lysozyme mRNA. The sequence was derived as outlined in Fig. 1*A* by sequencing pls-1 (17). The first 62 residues (arrow) were determined by cDNA extension on partially purified lysozyme mRNA (22). In the sequence of the structural parts of the lysozyme gene (exon sequences determined as outlined in Fig. 1*C*) only deviations from the pls-1 sequence are presented and those nucleotides that correspond to the extreme 5′ and 3′ ends of the mRNA. Numbering starts at the most likely position for the 5′ end of the mRNA (see text). Vertical bars denote canonical exon boundaries. Underlined bases in the 5′ noncoding region can pair with 18S RNA (26).

the codons for 18 amino acids that comprise the signal peptide of prelysozyme. The corresponding nucleotide sequence confirms the amino acid sequence of this extremely hydrophobic NH_2-terminal protein extension as determined by Palmiter *et al.* (31) and shows a stretch of 38 bases without A, which ends directly at the first codon for mature lysozyme. When we compared the structural gene sequence derived from genomic DNA clones with that derived from the cDNA clone we observed four base-pair transitions (Fig. 2, positions 175, 176, 400, and 434). Even though sequence variations have been shown to occur between allelic genes (32), we suspect that the transitions observed here may not represent true allelic variations. Because some of the transitions could cause amino acid changes, we think it is more likely that they originated all or in part during construction of the cDNA plasmid. For codon 103, however, we determined the sequence AAC (Asn) in both the cloned cDNA and in the isolated gene DNA. This nucleic acid sequence result settles the uncertainty (33) about the true identity at this position (ref. 34, no. 104 in alignment 12).

Exon/Intron Structure of the Lysozyme Gene. Electron microscope and biochemical mapping experiments (14–16, 35) have revealed that the lysozyme gene sequence is 6–7 times longer than its corresponding mRNA sequence and that it contains at least three intervening sequences. To elucidate the precise sequence organization of the gene, we localized all exon boundaries and determined the sequence of their immediate flanking regions according to the strategy outlined in Fig. 1*C*. Extensive sequence data from intervening and 5′- and 3′-flanking gene DNA will be published elsewhere.

We found that the complete structural gene sequence (equivalent to mRNA sequence) in the cloned gene DNA was interrupted not more than three times by intervening sequences (Fig. 1*B*). Fig. 3 shows the sequences flanking the six exon/intron boundaries of the chicken egg white lysozyme gene. The sequences at the exon/intron junctions satisfy the 5′ G-T···A-G 3′ rule (36) for the 5′ and 3′ ends of introns in eukaryotic mRNA specific genes. The boundaries were lined up according to this rule. They bear strong resemblance to the consensus sequences at the splice junctions of heterogeneous nuclear RNA (36, 37). Intron 1 has two possibilities and introns 2 and 3 have three possibilities for establishing the correct reading frame after splicing. Repeats directly at the junctions never exceed more than three base pairs. However, it is remarkable that the sequence 5′ T-G-A-G-C-T-C-A-G 3′ including a *Sac* I site starting at position 2 of intron 2 is repeated at the beginning of exon 3 starting position 7 (Fig. 3). Such a sequence repeat of nine base pairs would not be expected to occur statistically in a DNA stretch shorter than about 70 times the length of the complete lysozyme gene. Another direct sequence repeat is framing either side of the 3′ terminus of intron 1.

The most interesting result is derived from the location of the exon boundaries when correlated to the amino acid sequence of lysozyme. All three introns interrupt the coding sequence for mature lysozyme. The gene for lysozyme does not possess a mRNA leader sequence as found in other genes (36, 38–40). Nor is there any interruption of the 3′ noncoding region in the gene as we had deduced earlier from analysis of total genomic DNA by Southern hybridization (13). All three conventional exon boundaries lie within codons (Fig. 3). Intron 1 interrupts codon 28 (Trp). Intron 2 interrupts codon 82 (Ala) with a second splicing alternative between codons 82 and 81 (Ser) and a third alternative within codon 81. Intron 3 interrupts codon 108 (Trp), with one of the three splicing alternatives between codons 108 and 109 (Val). The significance of the locations of the introns with respect to structure and function of the coded protein and the implications of the gene structure for the evolution of the lysozyme gene will be considered in the discussion.

DISCUSSION

Few proteins are as well known as chicken lysozyme. Shortly after its primary structure was determined (41, 42) it became the first enzyme for which the three-dimensional structure was elucidated (43). This led to a detailed explanation of its catalytic properties and was a hallmark for our understanding of enzyme function in general (for review see ref. 33). Determination of the exact position of the exon/intron boundaries makes it pos-

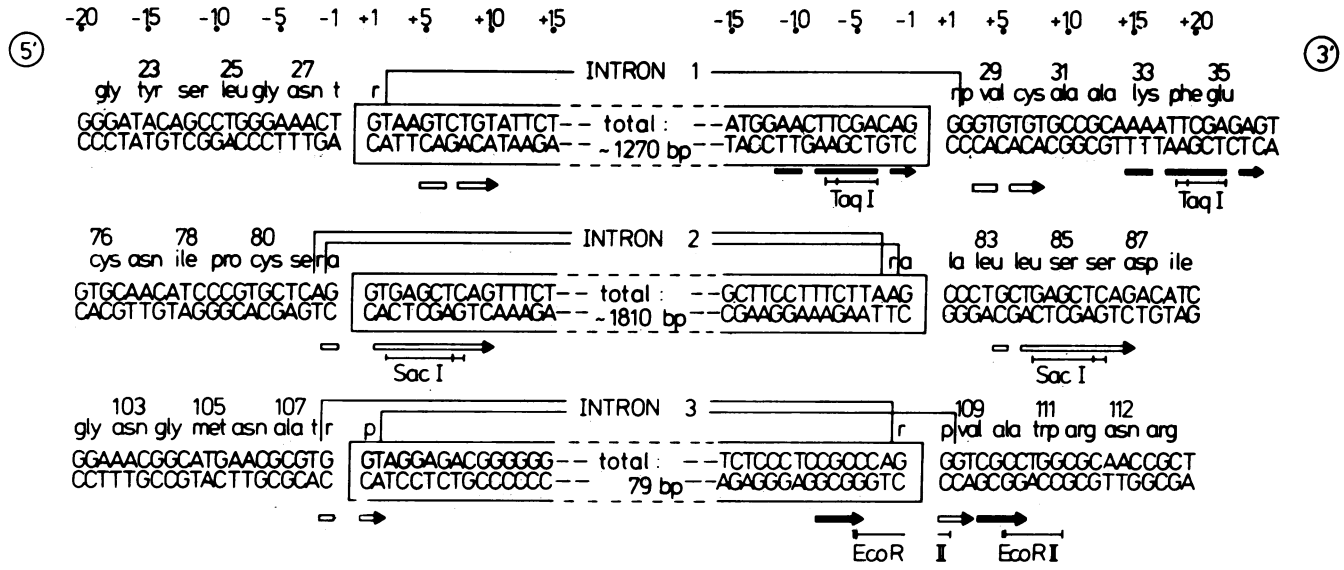

FIG. 3. DNA sequence of exon/intron boundaries of the lysozyme gene. Sequences were determined as outlined in Fig. 1*C* and compared with the lysozyme mRNA sequence (Fig. 2) for determination of the exact exon/intron boundaries. The three intron sequences are boxed according to the 5′ G-T···A-T 3′ rule (36); alternative splicing frames are indicated by brackets. Underlining open bars denote direct repeats located close to the 5′ end of introns and to the 5′ ends of the following exon. Solid bars denote direct repeats close to the 3′ end of introns and to the 5′ end of the following exon.

sible to correlate the structure of the gene to structure and function of the protein (Fig. 4).

On the basis of structural considerations, protein chemists have divided lysozyme into four segments: (*i*) residues 1–39; (*ii*) 40–85; (*iii*) 86–100; and (*iv*) 101–129 (43–47). A deep crevice containing the active site divides the molecule into two halves. On one side of the crevice is the β-structured second segment and on the other side are the two chain-terminal α-helical segments. The third segment with a regular α-helical element from residues 88–99 joins these two halves. After the preliminary characterization of the gene by Southern blotting and electron microscopy it was tempting to associate these structural segments with the detected four coding sequences. With the exact exon determination by DNA sequence analysis we found that this correlation is not as close as was suspected. Intron 1 interrupts at codon 28 within the sequence for the α-helix 24–34 and does not interrupt around codon 40. However, with the exception of this first exon/intron boundary there is a reasonable correlation of the exon pattern and the compact structural units of the enzyme molecule. Each of the products of the four exons carries a main structural element which folds independently and does not penetrate very much into other exon units.

The deviation at the first exon/intron boundary should be seen in the context of function of the enzyme molecule. Exon 2, which codes for Trp-28 to Ala-82, keeps together the catalytic center of the enzyme with Glu-35 and Asp-52 and the surrounding functionally important elements from both sides of the crevice, the antiparallel pleated sheet and the near part of the opposite helix 24–34. Fig. 4 shows, in a two-dimensional way, the amino acids that make contact to the hexasaccharide substrate as deduced from x-ray crystallography and model building (33, 48). One cluster of contacts to the substrate is formed by the peptide part coded on exon 2. These interactions occur exclusively with the carbohydrate rings C, D, E, and F and are therefore next to the β-1,4-glycosidic bond between rings D and E which is the target of the catalytic action of the enzyme.

The product of exon 3 completes the front part of the second lip of the crevice which is still left open by the product of exon 2. With this formation, exon 3 specifies a second cluster of amino acids that interact with the substrate. The product of exon 3 contributes to the structure of the specificity site (site C). It excludes *N*-acetylmuramic acid from position C by steric hindrance and thus determines the specific cleavage frame of the alternating *N*-acetylglucosamine/*N*-acetylmuramic acid copolymer (49). Intron 3 interrupts the coding sequence at the very beginning of the α-helix 108–115. Trp-108 contributes to the hydrophobic environment around Glu-35 and at the same time is an important residue for the specificity of substrate binding. One of the functionally important centers of the enzyme is therefore determined by a nucleotide sequence in the immediate vicinity of a RNA splice point.

Within the protein product of exon 4 only Val-109, with an unclear function, and Arg-114, due to its long side chain, contribute unessentially to substrate binding (33). The peptide part of lysozyme encoded by exon 1 does not contain any contacts to the substrate. The two chain-terminal exon products are in close proximity to each other. They are connected by a disulfide bond and stabilize the enzyme molecule. Other functions can not be assigned to them at the moment. However, the full biological significance of lysozyme is not yet understood. There are indications for an additional nonantimicrobial function (50) and it is therefore possible that exon 1 and 4 products are in-

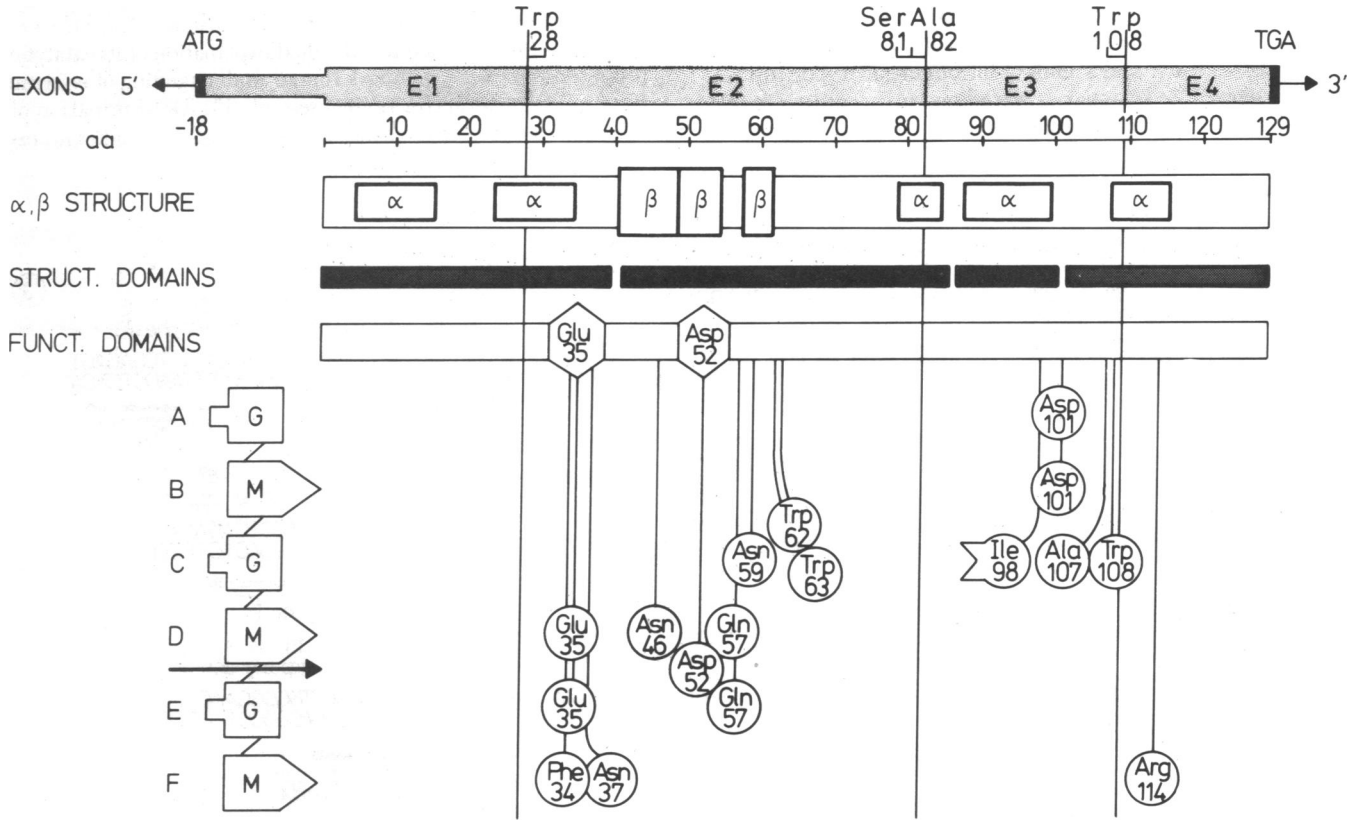

FIG. 4. Correlation between structural and functional parts of lysozyme and the exons of its gene. A linear diagram is shown of the structural gene parts encoded by exons, the α-helical and β-sheet structural elements (33, 43), and the structural segments of the protein that have been described (43–47). Functionally important residues of the enzyme are delineated two-dimensionally in correlation with their contact points in ring positions A–F of the substrate, an alternating copolymer of *N*-acetylglucosamine (G) and *N*-acetylmuramic acid (M).

volved in those. From evolutionary considerations one may assign more importance to exons 1 and 4 as the necessary DNA-, RNA-, and peptide signal environment for the transcription, translation, and transport of the catalytic functions carried on exons 2 and 3. Regulation at the lysozyme gene must be complex. Exon 1 connects the first coding section for the mature protein uninterruptedly to the promoter region of the gene (Fig. 1). In the oviduct cells the activity of the lysozyme gene is regulated by steroid hormones (35, 51, 52) but lysozyme is also expressed in macrophages independent of hormonal control (unpublished data).

We do not know how the exon/intron structure of the lysozyme gene was formed during evolution. The exon pattern shows, however, several aspects that support the idea that the lysozyme gene was built by adding exons to one another. Structural considerations favor processes that combine topologically compact units, in lysozyme best represented by the products of exon 1 and 3. The overall structure is stabilized when these units are joined by stiff connections. In lysozyme as in hemoglobin (6), all introns interrupt coding sequences for α-helical structures. Operational considerations favor processes that combine separately functioning units. Exon 2 could well represent a primitive glycosidase to which substrate specificity was confered by the product of exon 3. Comparative studies of the organization of other lysozyme related genes and their protein products might elucidate the steps that led to such a case of functional complementation by exons within one transcriptional unit.

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