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Cloning the human lysozyme cDNA: Inverted *Alu* repeat in the mRNA and *in situ* hybridization for macrophages and Paneth cells

(cDNA sequence/lysozyme expression/macrophage secretion product)

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ABSTRACT **Lysozyme is a major secretory product of human and rodent macrophages and a useful marker for myelomonocytic cells. Based on the known human lysozyme amino acid sequence, oligonucleotides were synthesized and used as probes to screen a phorbol 12-myristate 13-acetate-treated U937 cDNA library. A full-length human lysozyme cDNA clone, pHL-2, was obtained and characterized. Sequence analysis shows that human lysozyme, like chicken lysozyme, has an 18-amino-acid-long signal peptide, but unlike the chicken lysozyme cDNA, the human lysozyme cDNA has a >1-kilobase-long 3′ nontranslated sequence. Interestingly, within this 3′ region, an inverted repeat of the *Alu* family of repetitive sequences was discovered. In RNA blot analyses, DNA probes prepared from pHL-2 can be used to detect lysozyme mRNA not only from human but also from mouse and rat. Moreover, by *in situ* hybridization, complementary RNA transcripts have been used as probes to detect lysozyme mRNA in mouse macrophages and Paneth cells. This human lysozyme cDNA clone is therefore likely to be a useful molecular probe for studying macrophage distribution and gene expression.**

Lysozyme (EC 3.2.1.17) is a muramidase, which hydrolyzes 1,4-β linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine in the bacterial cell wall. Lysozyme has been extensively used as a model system for a large number of important studies (1), notably x-ray crystallography for protein structure determination (2) and mechanism of enzyme action (3). It is well known that lysozyme is synthesized by certain glandular epithelial cells and some leukocytes. By screening a large number of human and mouse cell lines with characteristics of T lymphocytes, B lymphocytes, lymphomas, and myeloma, only cells from the myelomonocytic lineage were shown to synthesize lysozyme (4). Since granulocytes and macrophages are probably a major source of lysozyme found within tissues, lysozyme can act as a very useful specific marker for the myelomonocytic lineage in normal and disease states. Indeed, increased levels of lysozyme in urine and serum are diagnostic indicators for acute monocytic leukemia and acute myelomonocytic leukemia (5). Moreover, the differentiation of myeloid cells could be studied by their ability to synthesize lysozyme in response to various inducers (6).

We are interested in investigating various aspects of the control of gene expression and secretion by macrophages and using lysozyme as a tool. Lysozyme is one of the most abundant secretory products of macrophages, representing up to 2.5% of the total protein synthesized *in vitro* (7). Although amino acid sequence data on lysozymes from a large number of species are available, only a few have been cloned (1). For the phage-type lysozymes, genes of the *Escherichia coli* phage T4 (8), *Salmonella* phage P22 (9), and *Bacillus* phage φ29 (10) have been isolated. The invertebrate chicken-type lysozyme sequenced and cloned is from the Cecropia moth, *Hyalophora cecropia* (11). So far, the best studied vertebrate lysozyme gene is the chicken lysozyme gene (12). Among mammalian lysozymes, human, baboon, and rat lysozyme have been completely sequenced (1) at the protein level. In this report, we describe the cloning of the human lysozyme cDNA and show that this lysozyme probe can also serve as a very useful tool for studying macrophages in human and other species.*

MATERIALS AND METHODS

Oligonucleotide Probes. Based on the human lysozyme amino acid sequence (13), two regions were chosen for oligonucleotide probes on the basis of minimum redundancy in codon usage. The two oligonucleotide probes were synthesized by the solid-phase phosphotriester method (14). Probes 1 and 2, containing sequences complementary to all the possible combinations of codons for amino acids 27–32 (Asn-Trp-Met-Cys-Leu-Ala) and 63–68 (Tyr-Trp-Cys-Asn-Asp-Gly), respectively, were mixtures of 32 and 16 different 17-base-long oligonucleotides (the third base of the codon for the last amino acid was excluded). The sequence of probe 1 was [GC(ACGT)A(AG)(AG)CACATCCA(AG)TT] and that of probe 2 was [CC(AG)TC(AG)TT(AG)CACCA(AG)TA]. Both probes were complementary to the lysozyme mRNA sequence and radiolabeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase.

Screening of cDNA Library. The cDNA library used in this study was prepared from mRNA of phorbol 12-myristate 13-acetate (PMA)-induced U937 cells (15) by the RNase H method and was kindly provided by S. K. A. Law (Medical Research Council, Immunochemistry Unit, Oxford). The techniques for screening the cDNA library (16) and purification, restriction enzyme digestion, subcloning, and other manipulations of the cloned DNA (17) were as described.

RNA Blot Analysis. Cells were lysed in guanidinium isothiocyanate (18) and RNA was isolated by ultracentrifugation over a cesium chloride cushion. RNA samples were electrophoresed on a 1% agarose gel with 6% formaldehyde and blotted overnight onto nitrocellulose filter (19). The filter was hybridized with a cDNA probe prepared from a *Bam*HI/*Pst* I fragment (see Fig. 2) radiolabeled by random priming (20). The hybridization was carried out in the presence of 50% formamide/10% dextran sulfate. To detect lysozyme mRNA from other species, the filters were washed under lower stringency conditions (3× SSC at 60°C for 90 min; 1× SSC = 0.15 M NaCl/0.015 M sodium citrate).

***In Situ* Hybridization.** The *Bam*HI/*Rsa* I fragment of the cDNA (see Fig. 2) was blunt-ended and subcloned in both

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Abbreviation: PMA, phorbol 12-myristate 13-acetate.
*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03801).

orientations into the *Hinc*II site of pGEM-3 (Promega Biotec, Madison, WI). The recombinant plasmids were purified, linearized by *Hin*dIII, and transcribed with T7 RNA polymerase in the presence of ^{35}S-labeled UTP (400 Ci/mmol; 1 Ci = 37 GBq) to generate high specific activity RNA probes for *in situ* hybridization experiments. The transcript obtained from pGBR2.4 was complementary to the lysozyme mRNA and gave positive hybridization signals. The pGBR2.3 transcript had the same sequence as the lysozyme mRNA and was used as a negative control. Isolated cells were cultured on clean sterile slides overnight, nonadherent cells were washed off, and the slides were fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS). The slides were rinsed in PBS, dehydrated in ethanol, and stored dry at −70°C for up to a month. Preparation of tissue sections and *in situ* hybridization were performed essentially as described (21).

RESULTS

Cloning of the Human Lysozyme cDNA. Since the complete amino acid sequence of human lysozyme was known, using synthetic oligonucleotides as probes to screen a macrophage cDNA library was the strategy adopted. A clone, designated pHL-1, containing plasmids that hybridized to both probe 1 and 2 was isolated. DNA sequencing performed on both ends of the insert in pHL-1, by the chemical method of Maxam and Gilbert (22), confirmed that pHL-1 indeed contained the human lysozyme cDNA. The 5′ end of this cDNA insert contains a sequence corresponding to the 5′ nontranslated region, the signal peptide, and the coding sequence of human lysozyme mRNA. However, the 3′ end of pHL-1 was defective. To obtain a proper full-length clone, a *Bam*HI/*Pst* I restriction fragment of pHL-1 was used as a probe to rescreen the cDNA library. Two more clones, pHL-2 and pHL-3, were isolated and their 5′ and 3′ terminal sequences were determined. At the 5′ end, pHL-2 contains a 5′ nontranslated region 13 nucleotides shorter than that found in pHL-1 but, otherwise, the complete signal peptide and coding sequences. At the 3′ end of pHL-2, a polyadenylylation signal and a poly(A) sequence were found. pHL-3 has the same 3′ sequence as pHL-2 but was shorter by ≈350 bases at the 5′ end. Based on this information, pHL-2 was chosen for detailed analysis. The complete DNA sequence of the pHL-2 insert was determined (Fig. 1) by the Sanger dideoxynucleotide chain-termination method after subcloning into M13 (23). A map summarizing the structure of the cDNA is shown in Fig. 2.

Structural Features of the Human Lysozyme cDNA. The amino acid sequence predicted from the unique open reading frame in this cDNA clone agrees exactly with that determined for human lysozyme by amino acid sequencing. The termination codon is TAA and the polyadenylylation signal is AATTAAA, located 14 nucleotides upstream from the poly(A) tail. The translation initiation site can be determined unequivocally because (*i*) there is only one ATG codon located upstream and in-frame with the coding sequence and (*ii*) there are termination codons on all three reading frames 5′ to this unique ATG codon. The signal peptide is 18 amino acids long and the cleavage site by the signal peptidase is a glycine residue. The 5′ nontranslated regions in pHL-1 and pHL-2 are, respectively, 25 and 12 nucleotides long. To estimate the length of the 5′ nontranslated region of the human lysozyme mRNA, a primer extension experiment (results not shown) was performed. The result indicated that most, if not all, of the 5′ nontranslated region is present in pHL-1.

The most exceptional feature of the cDNA sequence is the presence of an inverted *Alu* repeat (24) in the long 3′ nontranslated region. This repeat is 315 nucleotides long, spanning nucleotides 740–1054. The human lysozyme *Alu* repeat has all the characteristics of this family of repetitive sequences (Fig. 3) and shows 93% sequence identity with the recently compiled *Alu* repeat consensus (25). The A-rich sequences at the end of the first and second monomers are A_5TACA_6 and A_{12}, respectively. The terminal direct repeats are 13 nucleotides long and, apart from one nucleotide, the two repeats (AAGAACT/GGAATGT) are identical.

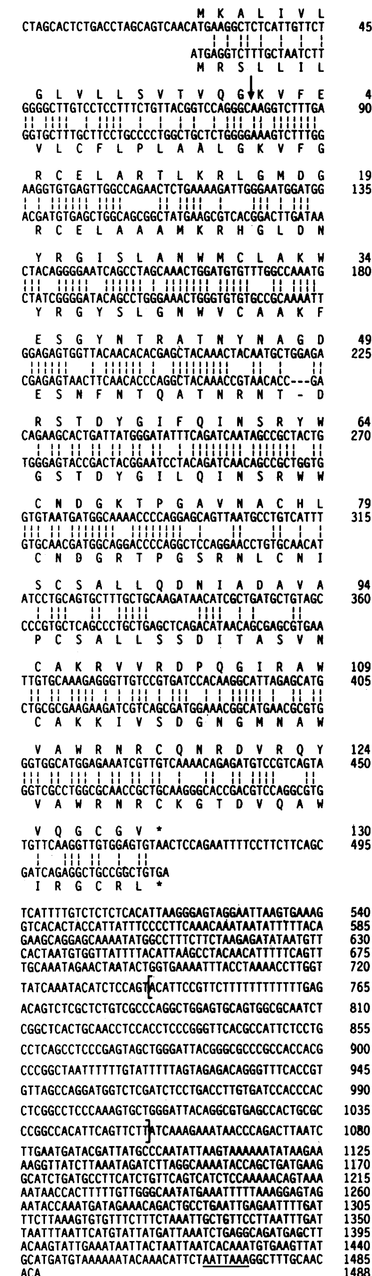

FIG. 1. The nucleotide sequence of the human lysozyme cDNA and comparison with the coding region of the chicken lysozyme cDNA. The first sequence (top line) is the human lysozyme cDNA and the second one (bottom line) is from the chicken lysozyme (12). Amino acids are designated by the single-letter code. Arrow marks site of the signal peptide cleavage. The polyadenylylation signal is underlined and brackets show the ends of the *Alu* repeat. Apart from the first 13 nucleotides, which are only present in pHL-1, the sequence shown is obtained from pHL-2. The strategy used was to sequence random fragments prepared by sonication of the self-ligated pHL-2 insert. Identical nucleotides in the human and chicken sequence are indicated by vertical lines. - - -, Gap introduced at amino acid residue 48 to allow maximum alignment of the two sequences; the human lysozyme (130 residues) is one amino acid longer than the chicken lysozyme (129 residues).

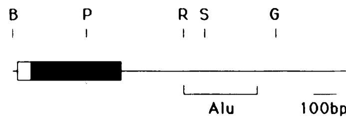

FIG. 2. A diagramatic representation of the human lysozyme cDNA. The signal peptide and the mature protein are denoted by an open box and a solid box, respectively. The nontranslated sequences are shown by a line. The position of the inverted *Alu* repeat in the 3′ nontranslated region is indicated. The unique cleavage sites by the restriction endonucleases *Pst* I (P), *Rsa* I (R), *Sma* I (S), *Bgl* II (G), and *Bam*HI (B) are marked. The *Bam*HI cuts within the vector at a position close to the cloning site and is useful for generating cDNA fragments from the clones. bp, Base pairs.

The Lysozyme mRNA. Based on the RNA blot analysis (Fig. 4), the human lysozyme RNA appeared to migrate slightly faster than the 18S RNA marker. With radiolabeled *Hin*dIII fragments of bacteriophage λ DNA as marker, human lysozyme RNA was estimated to be ≈1.6 kilobases, which is in close agreement with the size determined by sequencing the cDNA clone. Often, a minor band of ≈3.8 kilobases is also observed, the identity of which is unknown, but it may represent incompletely spliced lysozyme transcript. Although the lysozyme mRNA was detected in both the human promyelomonocytic line HL-60 and the human monocytic line U937, the strongest signal was obtained from cultured human monocytes. In fact, hybridization signals have been obtained from <2 μg of total RNA of cultured human monocytes after 4 hr of exposure, indicating that the lysozyme probe can be used as a sensitive reagent for detecting macrophage RNA. The level of lysozyme mRNA was slightly increased when HL-60 cells were treated with dimethyl sulfoxide, which induced them to differentiate into the granulocytic phenotype, but was decreased when the cells were treated with PMA, which induced them to differentiate into a macrophage-like phenotype (results not shown). Moreover, untreated U937 cells had more lysozyme mRNA than HL-60 cells, regardless of whether the HL-60 cells were treated with dimethyl sulfoxide and PMA. However, if the U937 cells were treated with PMA for 2 days, the

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1056   ATAAGAACTG AATGTGGCCG GGCGCAGTGG CTCACGCCTG
                            *      **
                      GGCTG GGCGTGGTGG CTCACGCCTG

1016   TAATCCCAGC ACTTTGGGAG GCCGAGGTGG GTGGATCACA
                                                *
       TAATCCCAGC ACTTTGGGAG GCCGAGGTGG GTGGATCACC

976    --AGGTCAGGAG ATCGAGACCA TCCTGGCTAA CACGGTGAAA
       **           *  *       *      *     *
       TGAGGTCAGGAG TTCAAGACCA GCCTGGCCAA CATGGTGAAA

936    CCCTGTCTCT ACTAAAAATA CAAAAAATTA GCCGGGCGTG
          *                      *
       CCCCGTCTCT ACTAAAAATA CAAAAA-TTA GCCGGGCGTG

896    GTGGCGGGCG CCCGTAATCC CAGCTACTCG GGAGGCTGAG
             *      *
       GTGGCGCGCG CCTGTAATCC CAGCTACTCG GGAGGCTGAG

856    GCAGGAGAAT GGCGTGAACC CGGGAGGTGG AGGTTGCAGT
                  *  *
       GCAGGAGAAT CGCTTGAACC CGGGAGGTGG AGGTTGCAGT

816    GAGCCGAGAT TGCGCCACTG CACTCCAGCC TGGGCGACAG
                  *
       GAGCCGAGAT CGCGCCACTG CACTCCAGCC TGGGCGACAG

                              ====== ======
776    AGCGAGACT- -GTCTCAAAAAA AAAAAAGAAC GGAATGTAC   738
                *  *
       AGCGAGACTC CGTCTC
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FIG. 3. The *Alu* repeat in human lysozyme cDNA. The first (top) sequence is the reverted *Alu* repeat in the human lysozyme cDNA—that is, the complementary sequence of nucleotides 738–1056. The second sequence is the *Alu* consensus described (25). The differences in the two sequences are highlighted by asterisks and the terminal direct repeats are underlined. The characteristic A-rich sequence marking the end of the first monomer is overlined and = = = shows the A-rich sequence at the end of the second monomer.

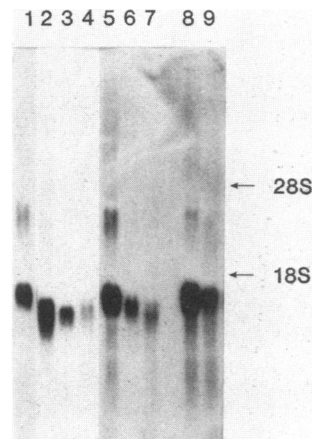

FIG. 4. RNA blot analyses. Lanes: 1, 3-day cultured human monocytes (3 μg); 2, 3-day cultured mouse thioglycollate elicited macrophages (3 μg); 3, 2-hr cultured rat thioglycollate elicited macrophages (3 μg); 4, mouse bone marrow cells that contain mostly immature granulocytes (5 μg); 5 and 8, human monocytic line U937 (10 μg); 6, mouse macrophage line J774 (20 μg); 7, the putative rat macrophage line R2 (20 μg); 9, 2-day PMA-treated U937 (20 μg). Different exposure times were used for these samples on the same blot. The positions of the 28S and 18S RNA are indicated by arrows.

lysozyme mRNA detected was significantly reduced (Fig. 4, lanes 8 and 9).

Apart from human lysozyme RNA, both the mouse and rat lysozyme RNA can also be detected in RNA blots by DNA probes prepared from the human lysozyme cDNA under low stringency conditions. The mouse and rat lysozyme RNA is ≈250 and ≈350 bases smaller, respectively, than that of the human. So far, using the human lysozyme cDNA probe in RNA blot analyses, detectable hybridization signals have been obtained from human monocytes, U937, HL-60, mouse peritoneal macrophages, mouse spleen macrophages, mouse bone marrow, J774, rat peritoneal macrophages, and the putative rat macrophage line R2 (26), but not from human endothelial cells, HeLa cells, mouse dendritic cells, mouse fibroblasts, mouse keratinocytes, and a number of T- and B-cell lines from human, mouse, and rat.

***In Situ* Hybridization.** Apart from macrophages, it is well known that epithelial cells, such as the Paneth cells, also produce large quantities of lysozyme. Fig. 5 *D–F* shows hybridization of the lysozyme probes to small bowel sections. The results confirm earlier observations using immunocytochemistry that Paneth cells, in intestinal crypts, are major lysozyme producers (27). As examples of mouse macrophages synthesizing lysozyme, *in situ* hybridization on cultured mouse thioglycollate-elicited peritoneal macrophages and alveolar macrophages in a lung section are shown (Fig. 5). As a further specificity control, we have used thioglycollate-elicited peritoneal macrophages cocultured with HeLa cells. Further details of *in situ* hybridization on other tissues will be presented elsewhere.

DISCUSSION

The use of monoclonal antibodies, such as F4/80 (28), to localize macrophages in tissues has been very fruitful in studies on the distribution of macrophages under normal and disease states. However, most monoclonal antibodies are species restricted and therefore cannot be used in other animal models. Unlike the approach of using monoclonal antibodies, nucleic acid probes specific for one species can often be used in other species as well. Lysozyme has been shown to be a tissue-restricted marker for myelomonocytic cells, and macrophages cultured *in vitro* under many different

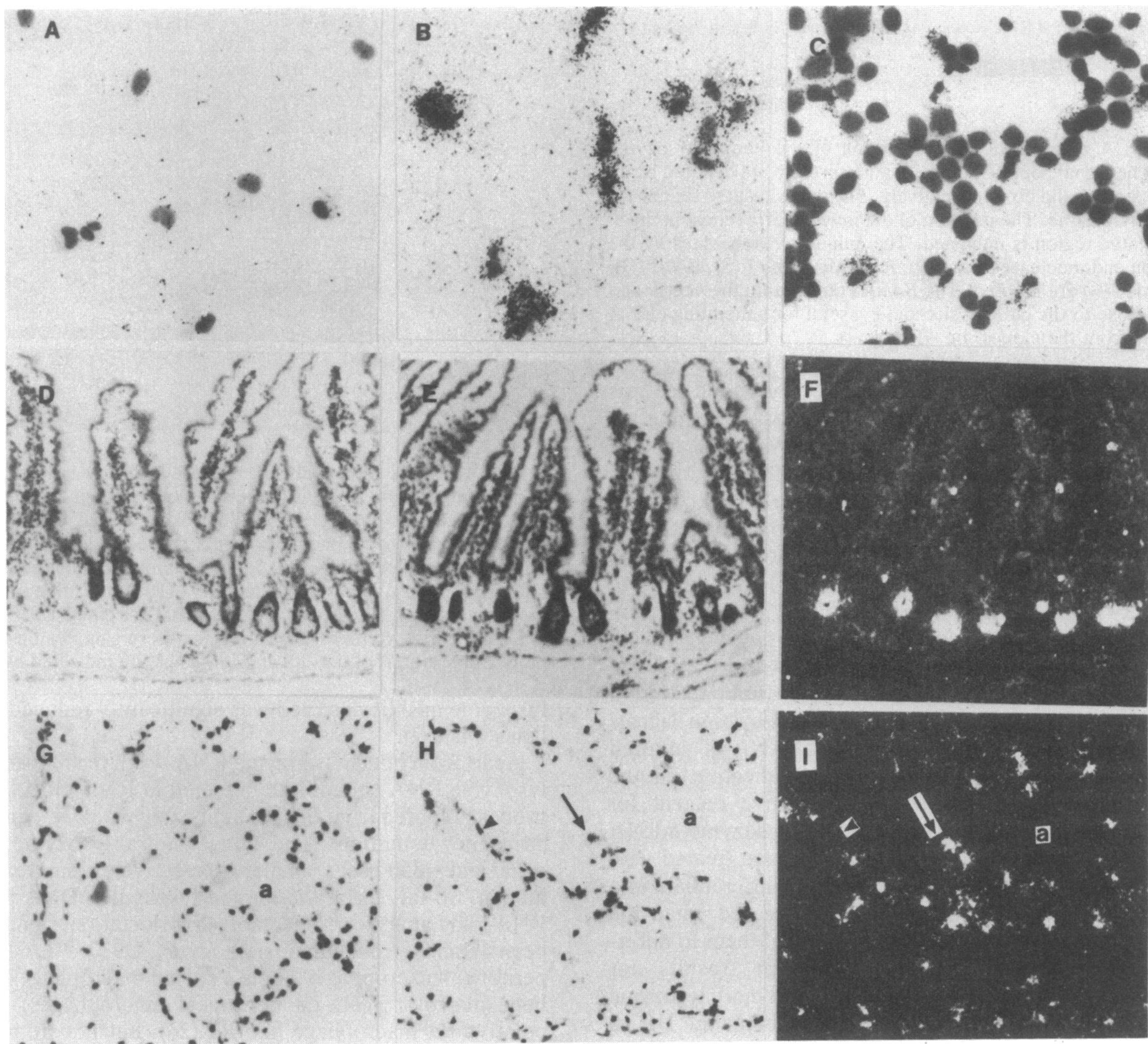

FIG. 5. Detection of lysozyme mRNA by *in situ* hybridization. (*A*, *D*, and *G*) Hybridized with probe pGBR2.3 (sense strand, negative control). (*B*, *C*, *E*, and *H*) Hybridized with probe pGBR2.4 (antisense strand, complementary to lysozyme mRNA). (*F* and *I*) Dark-field micrographs corresponding to *E* and *H*. (*A* and *B*) Thioglycollate-elicited peritoneal macrophages cultured overnight on glass slides show a strong specific signal. (*C*) Peritoneal macrophages cocultured with HeLa cells show strong hybridization, while HeLa cells are unlabeled. (*D–F*) Sections of mouse small bowel show cells in the base of intestinal crypts hybridizing strongly to the antisense probe. The positive cells correspond histologically to Paneth cells. (*G–I*) Sections of mouse lung show macrophages in the walls of alveoli (a) hybridizing to the antisense probe (e.g., arrow). The positive cells are disposed among numerous nonmacrophage negative cells in the alveolar wall (e.g., arrowhead). *A–C* were exposed for 6 days and the rest were exposed for 3 days.

conditions have been shown to produce relatively large quantities of lysozyme (29). We have therefore chosen to use lysozyme as a tool for *in vivo* macrophage localization, as well as a model for studying molecular mechanisms involved in the control of gene expression and secretion in macrophages. Through database searches, we have not been able to find reports on cloning of any mammalian lysozyme. Moreover, in our hands, the chicken lysozyme cDNA probe does not cross-hybridize to human or mouse lysozyme mRNAs. Thus, as a first step in our studies, cDNA clones of the human lysozyme were isolated and characterized.

The Human Lysozyme cDNA. Sequence analysis on pHL-2, the full-length cDNA clone isolated, shows that apart from the long 3′ nontranslated sequence, the human lysozyme cDNA has considerable structural similarity with the chicken cDNA. Fig. 1 shows the comparison of the chicken and human lysozyme cDNA. For the signal peptides, which are both 18 amino acids long, there is 53% similarity both at the level of DNA and amino acid (29% identity and 24% conserved changes). While the mature proteins show a total of 73% similarity (61% identity and 12% conserved changes), there is 61% similarity between the two cDNAs.

The Human Lysozyme mRNA Contains an Inverted *Alu* Repeat. Most *Alu* sequences have been found in introns or intergenic regions and only very few have been shown to be present in mature mRNA molecules, although *Alu* sequence-containing cytoplasmic RNAs are known to be present (30). Like all other repetitive sequences, the functional significance of this inverted *Alu* repeat in the human lysozyme mRNA remains to be determined. Nevertheless, from the evolutionary point of view, it would be interesting to know whether *Alu* or *Alu*-like repetitive sequences can be found in the lysozyme mRNA of other primates and mammals, considering the potential effects of these repetitive sequences in reorganizing eukaryotic genomes. The chicken lysozyme gene region has been shown to contain a complex array of at least five classes of repetitive, but probably not *Alu*-like, sequence (31), and in the human α-lactalbumin gene (which is structurally and functionally related to lysozyme), an *Alu* repeat has been found in the first intron (32).

***In Situ* Hybridization.** Despite the detailed structural studies on lysozyme, relatively little is known about the biosynthesis and cell source of lysozyme in the animal. We have demonstrated that the lysozyme probe is useful in studying the distribution and identification of tissue macrophages and other lysozyme-secreting epithelial cells, not only in human, but also in other mammals including mouse and rat. It is known that serum lysozyme levels increase in patients with Crohn disease and sarcoidosis, granulomatous diseases in which macrophages are prominent. Therefore, the lysozyme probe could be useful to define the source of lysozyme and the regulation of macrophage activity in these and other inflammatory diseases.

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