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Mouse lysozyme M gene: Isolation, characterization, and expression studies

(cDNA/macrophage/lysozyme P/tissue specificity/α-lactalbumin)

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ABSTRACT **We have isolated and characterized both cDNA and genomic DNA of the mouse lysozyme M gene. Derivation of the amino acid sequence from the nucleotide sequences revealed six positions in the carboxyl terminus that differ from partial sequences previously published. The differential detection of specific mRNAs from the closely related lysozyme M and P genes has revealed different but overlapping tissue specificities of expression. The M gene is expressed weakly in myeloblasts, moderately in immature macrophages, and strongly in both mature macrophages and macrophage-rich tissues, while high levels of P transcripts are present only in small intestine. Sites of protein accumulation, rather than gene expression, have been identified by comparative quantitation of mRNA and enzyme levels.**

Since the discovery in 1922 of lysozyme (EC 3.2.1.17) via its antibacterial effects (1), the enzyme has been adopted as a model for studies on protein structure and molecular evolution (for review, see ref. 2), while the lysozyme gene has been the subject of investigations into the determinants of tissue specificity and hormonal control of gene expression (3–6). Comparison of lysozyme sequences with those of α-lactalbumin have implied an evolutionary relationship between these proteins (7), a hypothesis that has been strengthened by the demonstration of strong similarities within the crystal structures of the proteins (8) and the sequences and arrangements of their respective genes (9). The involvement of lysozyme in host defense has prompted numerous studies of the tissue distribution of lysozyme enzyme activity in animals in both normal and pathogenic states. Hence, deviations in lysozyme levels of blood and urine, for example, have been correlated to particular myeloid and renal abnormalities, and lysozyme levels have subsequently been used to monitor the success of therapy (10, 11). Although the lysozyme protein can be found in most tissues, studies at the cellular level have revealed high levels of lysozyme specifically in phagocytic cells, including macrophages, granulocytes, and the Paneth cells of the small intestine, and in the proximal tubules of kidney (12, 13). While the kidney accumulates at least some of its lysozyme from the blood (14), endogenous lysozyme synthesis has been detected in mammalian myeloid cells (15). The recent discovery of high levels of intestinal lysozyme in some species of house mice, in which the enzyme may have assumed a digestive role, has recently stressed the involvement of regulatory mutations in evolution (16, 17). In this case, a regulatory mutation appears to have affected only one of two closely related lysozyme genes, resulting in a specific overexpression of lysozyme P in the small intestine and leaving normal expression of lysozyme M in other tissues tested.

Studies on the expression of the chicken lysozyme gene have described particular DNA sequences that demonstrate a positive or negative effect on gene expression in a macrophage-specific manner (4–6) and others that bind steroid hormone receptors and mediate hormonal control in oviduct cells (3). Our intention is to extend studies of lysozyme gene regulation to the mouse, from which the availability of a number of myeloid cell lines representing different stages of myeloid differentiation and the possibility of generating chimeric or transgenic animals present an extremely versatile system. We present here the isolation and characterization of the mouse lysozyme M gene.† We have determined the tissue distribution of both lysozyme protein and mRNA, permitting a distinction to be made between sites of gene expression and protein accumulation, and describe a strong dependence of levels of lysozyme gene expression on the stage of differentiation of myeloid cells. Successful resolution of the mRNAs from the lysozyme M and P genes has facilitated independent determinations of their tissue specificities of expression.

MATERIALS AND METHODS

Isolation of Mouse Lysozyme M cDNA and Genomic Clones. The spleen of a mouse (*Mus domesticus*) sacrificed 14 days after infection with AF1 virus (18) was kindly provided by W. Ostertag (University of Hamburg, Hamburg, F.R.G.). Polyadenylylated RNA was purified from this tissue by the LiCl precipitation method (19) and subsequent selection on oligo(dT)-cellulose. A cDNA bank was constructed in λgt10 by the method of Gubler and Hoffman (20). A screen of duplicate plaque lifts on GeneScreen membranes (New England Nuclear) was performed under the manufacturer's recommended conditions with nick-translated probes from part of a human lysozyme cDNA, kindly provided by P. Swetly (Ernst Boehringer Institute, Vienna). Hybridization was carried out in 40% formamide/0.75 M NaCl/75 mM sodium citrate at 50°C.

Fractions of a mouse (*M. domesticus*; t^{AE5}/1295v) gene bank in vector λEMBL3, kindly provided by B. Hermann (European Molecular Biology Laboratory, Heidelberg), were screened with nick-translated probes from the isolated mouse lysozyme M cDNA sequences. Hybridization was performed at 42°C in 50% formamide/0.75 M NaCl/75 mM sodium citrate. All standard DNA manipulations were carried out according to Maniatis *et al.* (21) unless otherwise indicated.

Sequencing. Plasmid DNA sequencing by the chain-termination method (22) was performed with a Sequenase kit (United States Biochemical, Cleveland). All reported sequences were determined independently from both DNA strands. Sequence analysis was performed with software from the University of Wisconsin Genetics Computer Group.

Analysis of Mouse Lysozyme mRNA. RNA prepared from cell lines or *M. domesticus* BALB/c tissues by the guanidinium isothiocyanate method was electrophoresed through

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†This sequence is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03846).

1.2% agarose/2.2 M formaldehyde denaturing gels (21), with RNA molecular weight markers (Bethesda Research Laboratories). RNA was blotted to GeneScreen membranes, which were subsequently stained with methylene blue to confirm quantitative transfer (23). Comparative quantitation of lysozyme-specific message in samples of total RNA was achieved by dot blot titration on GeneScreen membranes under conditions recommended by the manufacturer. Hybridization to nick-translated probes was carried out in 50% formamide/1 M NaCl at 42°C. Primer extension reactions were performed by the method of Ghosh *et al.* (24) with a 32-nucleotide primer complementary to part of the mouse lysozyme M mRNA. Sequencing reaction mixtures primed with the same oligonucleotide were used as size markers. Oligonucleotides for both primer extension and sequencing were synthesized in the laboratory of R. Mertz (Max Planck Institut Genzentrum, Martinsried, F.R.G.).

RNA·DNA hybrid analysis by S1 nuclease digestion was carried out essentially according to Weaver and Weissmann (25). RNA·RNA hybrids were analyzed by RNase A/RNase T1 digestion (26).

Lysozyme Enzyme Assays. The determination of lysozyme activity was performed by the lysoplate assay of Osserman and Lawlor (27) with freshly frozen tissues ground under liquid nitrogen or with freeze–thaw cell lysates. Total protein concentrations were determined by using the protein assay reagent and protocol from Bio-Rad.

Cells. The mouse myeloblast cell line M1 (28); immature macrophage cell lines WEHI3 and WEHI3B (29); mature macrophage lines P388D1 (30), J774 (31), HA32, and HA38 (AF1 virus-transformed, a gift from W. Ostertag); and the fibroblast cell line Ltk$^-$ (32) were grown in Dulbecco's modified Eagle's medium supplemented with fetal calf serum to 20% (HA32 and HA38) or 10% (all others). Primary macrophages were derived from bone marrow by the method of Stewart (33) and were used after 14 days in culture on tissue culture unprepared Petri dishes.

RESULTS

Mouse Lysozyme M cDNA. Screening a fraction of a mouse spleen cDNA library with human lysozyme cDNA sequences yielded 37 strongly hybridizing plaques from ≈3 × 10^4 recombinants. All of these isolates subsequently examined were found to contain lysozyme M cDNA, the complete sequence of which is indicated in Fig. 1.

The mouse lysozyme M amino acid sequence derived from the cDNA sequence completes, and is in full agreement with, available protein sequence data corresponding to residues 1–49 of the mature protein, but it differs at 6 positions from the partial sequence of the carboxyl-terminal region derived from tryptic peptide composition and alignment studies (38). The revised amino acid sequence similarity to human lysozyme is 77.7%, and the similarity to chicken lysozyme is 56.9%. Comparison to other available protein sequences confirms the conservation of residues involved in the catalytic center (Glu-35, Asp-53, Asp-102) and in protein folding (Cys-6, -30, -65, -77, -81, -95, -116, and -128; for reviews, see ref. 39).

A primer extension reaction (Fig. 2 *Left*) using a 32-base primer complementary to nucleotides 57–88 indicated the true start site of transcription to be 7 nucleotides upstream of the end point of the isolated cDNA (Fig. 1). Hybridization of both mouse (Fig. 2 *Right*) and human lysozyme cDNA (data not shown) to RNA blots revealed the presence of a single band corresponding to a message of 1.5 kilobases (kb) in RNA from the mouse macrophage cell line J774, spleen and intestine, but not in RNA from the mouse fibroblast cell line Ltk$^-$. This suggests that mouse lysozyme mRNA has a poly(A) tail of ≈230 nucleotides.

Mouse Lysozyme M Gene. Screening a mouse genomic DNA bank with the isolated cDNA yielded five overlapping lysozyme M clones. The region covered by these clones includes the complete gene (5 kb), 5.5 kb of upstream, and 6.5 kb of downstream sequences. Detailed sequencing of all exons and exon/intron borders confirmed the identity of the lysozyme M gene and the presence, size, and exact positions of three introns, with no differences being found between cDNA and exon sequences. This information, plus the sequence of 395 bases upstream of the start site, is presented in Fig. 1. The positions of the three introns in the mouse lysozyme M gene correspond exactly to those in the chicken lysozyme gene (40). Sequence similarities of rat α-lactalbumin to mouse lysozyme M, chicken lysozyme, and rat lysozyme (ref. 41, protein sequence only) are presented in

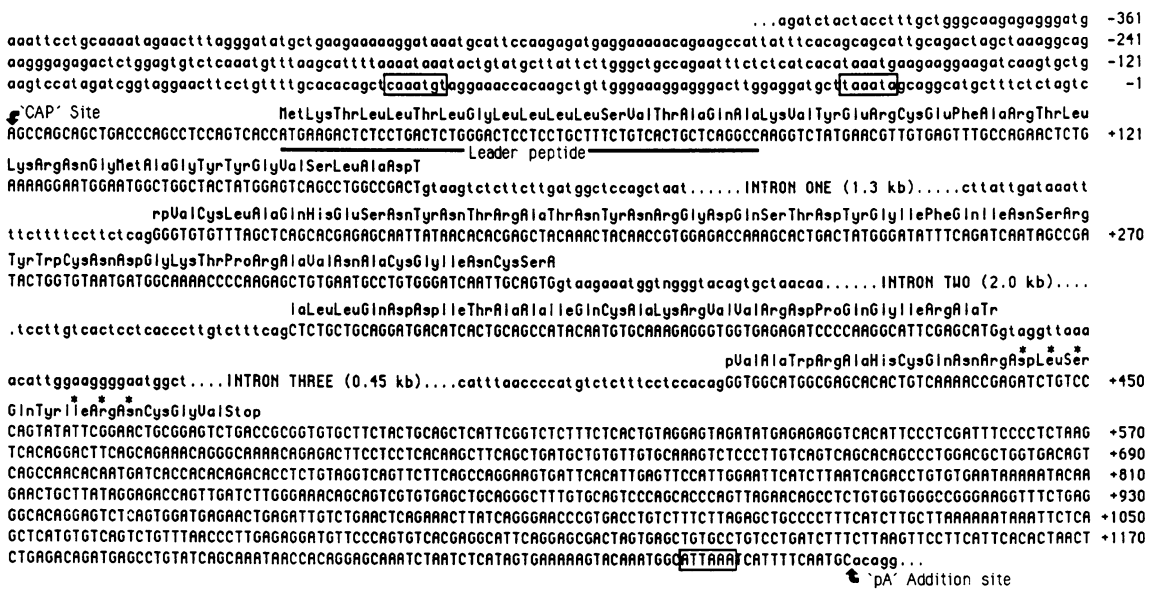

FIG. 1. Single (coding)-strand nucleotide sequence of the mouse lysozyme M gene and immediate upstream region. The cDNA sequence is indicated in capital letters, as are the 7 nucleotides between the end of the cDNA and the cap site (+1) as determined by primer-extension analysis. "TATA", "CAAT" (34, 35), and polyadenylylation signal (36) homologies are boxed. Introns are positioned according to the terminal GT–AG rule (37). Positions in the amino acid translation that differ from those previously published are indicated by an asterisk. Only upstream and cDNA sequences are numbered.

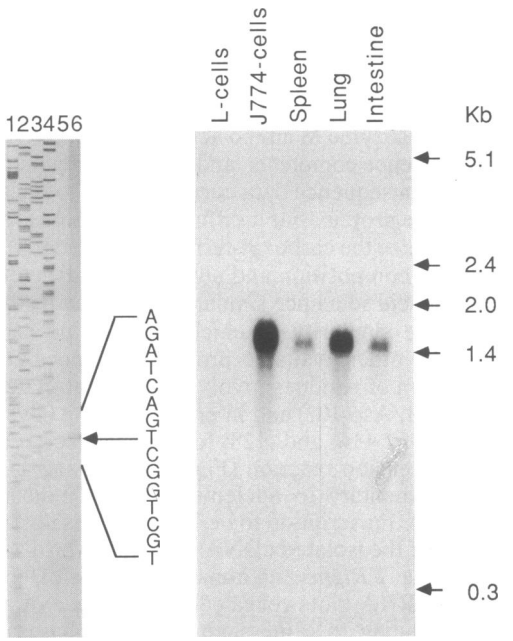

FIG. 2. (*Left*) Primer-extension analysis. (*Right*) RNA blot detection of mouse lysozyme M mRNA. (*Left*) Lanes: 1–4, sequence reactions of DNA spanning the transcription initiation region of the M gene; 5 and 6, 25:1 and 5:1 dilutions of a primer-extension reaction mixture performed with the same primer. The sequence of the noncoding strand is shown alongside, with an arrow indicating the position of the start site. (*Right*) Lysozyme M mRNA in 10-μg samples of total RNA was detected by hybridization to a lysozyme M cDNA probe from the region +8 to +776. The positions of stained RNA markers are indicated.

Table 1. A marked relatedness of the rat α-lactalbumin gene to the lysozyme genes is restricted to exons 1–3, with the exon 4 region being highly divergent.

Lysozyme Gene Expression in the Mouse. Expression studies of mouse lysozyme were carried out at both enzyme and mRNA levels to distinguish between sites of lysozyme gene activity and sites of accumulation of the protein. Comparative quantitation of lysozyme mRNA was achieved by dot blot titration of total RNA under probe hybridization conditions, which reproducibly demonstrated a single band on RNA blots. A summary of specific lysozyme mRNA levels and enzyme activity detected in selected tissues is presented in Table 2. In comparison to the fibroblast cell line used as a negative control, a low background level of both mRNA and enzyme activity was apparent in most tissues tested and is probably attributable to the presence of contaminating blood. The highest lysozyme mRNA levels were found in lung and bone marrow, while weaker expression was detected in small intestine, spleen, and thymus, and all other tissues contained specific mRNA levels close to background. In contrast, the tissues most rich in enzyme activity were kidney, spleen, and lung, with moderate levels in thymus and small intestine. Low levels were found in other tissues, brain being the only tissue tested in which no lysozyme activity was detectable. While it is possible that the lysozyme mRNA detected in lung, spleen, and thymus derives from myeloid cells present in these tissues, intestinal lysozyme expression has previously been attributed to the Paneth cells situated at the base of the crypts of Lieberkühn (12). Internal comparisons of mRNA and enzyme levels between different organs revealed an accumulation of disproportionately high concentrations of lysozyme protein in kidney, and to a lesser extent in spleen. These observations may be compared to an observed accumulation of radiolabeled blood lysozyme in these tissues in the rat (42).

Table 1. Nucleotide and amino acid sequence comparisons of rat α-lactalbumin to mouse lysozyme M, chicken lysozyme, and rat lysozyme

	Rat α-lactalbumin			
Lysozyme	Exon 1	Exon 2	Exon 3	Exon 4
Mouse M	51.2 (29.6)	53.9 (41.8)	54.4 (37.0)	38.8 (<4.8)
Chicken	52.4 (33.3)	54.3 (40.7)	46.8 (33.3)	35.8 (14.3)
Rat*	(29.6)	(40.0)	(37.0)	(9.5)

Percentage of positions having identical residues are shown; those at the amino acid level are in parentheses.
*Comparisons assume intron positions identical to those of mouse lysozyme M.

Table 2. Distribution of lysozyme in mouse cells and tissues

Cell type or tissue	Lysozyme activity, units per mg of protein	Lysozyme RNA, % primary macrophages
Fibroblast (L cells)	0	<<0.1
Myeloblast (M1 cells)	0.1	0.8
Immature macrophage (WEHI 3b cells)	<<0.1	0.3
Immature macrophage (WEHI 3 cells)	3	4.1
Mature macrophage (P388D1 cells)	ND	16
Mature macrophage (J774 cells)	30	82
Mature macrophage (HA 38 cells)	ND	114
Mature macrophage (primary culture)	ND	100
Brain	ND	<0.1
Liver	18	0.2
Kidney	4880	0.4
Salivary gland	5	0.4
Skeletal muscle	0	0.7
Pancreas	2	0.9
Stomach	14	0.9
Heart	5	1.4
Large intestine	3	1.7
Thymus	98	3.3
Spleen	1650	4.1
Small intestine	34*	12
Bone marrow	ND	33
Lung	1620	39

Enzyme activity is expressed relative to that of a chicken egg white lysozyme standard (SERVA, 24,000 units/mg). Lysozyme mRNA concentrations are presented as percentages of the level detected in mature primary macrophages. ND, not determined.
*Underestimated value, see text.

Primary macrophages and a range of myeloid cell lines were also tested for lysozyme expression to investigate possible correlations with the stage of macrophage development. In Table 1, the cell lines are arranged in order of increasing differentiation, after Leenen *et al.* (43). The levels of detectable lysozyme mRNA were very low in the myeloblast (M1) and one of the immature macrophage (WEHI 3B) cell lines. Another immature macrophage cell line (WEHI 3) demonstrated intermediate levels of expression, while the lysozyme gene was found to be strongly expressed in mature macrophages. The levels of specific mRNA were highest in primary macrophages and the AF1 virus-transformed macrophage cell line HA38. Although measurements of enzyme activity in cell lysates take no account of protein secretion, these were also found to increase with the stage of progression of macrophage differentiation.

Expression of the Lysozyme M and P Genes. In our lysoplate assays, there was a noticeable difference between the pattern of lysis mediated by extracts from intestine (clear plaques)

and those from other tissues and standard (turbid plaques; data not shown). We have also established that extracts from intestine have a characteristically low activity in lysoplate assays compared to liquid-phase spectrophotometric assays (results not shown). The values of lysozyme activity from small intestine are therefore underestimated and cannot be compared to the other values (Table 2). These observations are consistent with reports that lysozyme in the small intestine of mouse derives from a structural gene (the lysozyme P gene), separate from that encoding M lysozyme, and that the proteins differ in amino acid sequence at 6 of 49 positions near the amino terminus (16). The corresponding mRNAs proved to be inseparable on denaturing agarose gels (see Fig. 2 *Left*) and to be indistinguishable by hybridization to probes from either translated or untranslated regions of the M gene (data not shown). To examine further the expression of the two genes, we attempted to establish conditions allowing differential detection of the two types of transcripts to carry out independent determinations of their tissue distributions.

An S1 nuclease analysis of total RNA hybridized to a 5′-end-labeled DNA fragment overlapping the start site of the M gene indicated an identical pattern of protection of RNA from intestine, lung, and spleen (data not shown). The band intensities on the S1 nuclease map reflected the total lysozyme mRNA levels measured by dot blot hybridization at low stringency. Hence, the absence of different bands resulting from probe protection by lysozyme mRNA from intestine and that from other tissues is not likely to be a consequence of poor hybridization of lysozyme P mRNA to M DNA, but rather of sufficient homology between the molecules to form duplexes resistant to S1 nuclease digestion. The S1 nuclease analysis therefore suggests that the lysozyme P message encompasses a leader sequence that has high homology to that of the M message and is probably the same length. Hybridization of total RNA to *in vitro* transcribed RNA complementary to and overlapping the 5′ end of the lysozyme M message, followed by digestion with RNases A and T1, resulted in differential protection of M and P messages. This allowed the resolution of lysozyme P- and M-protected probe fragments on polyacrylamide denaturing gels. Using a variety of digestion stringencies we were able to establish conditions under which M (mRNA)/M (probe) hybrids were stably protected over a stretch of 166 nucleotides as expected (corresponding to the complete first exon), while P (mRNA)/M (probe) hybrids were completely degraded to smaller fragments, the largest of which was 96 nucleotides long (Fig. 3). Two mouse macrophage cell lines, as well as bone marrow-derived primary macrophages, were found to express very high levels of M lysozyme mRNA in the absence of detectable P lysozyme message. In contrast, RNA from small intestine demonstrated a vast excess of P over M message, consistent with the predominance of P lysozyme protein in this tissue (16). RNA from large intestine contained low levels of M lysozyme mRNA and no detectable P message. Testing RNA from a number of other tissues revealed very low levels of P lysozyme mRNA only in lung, thymus, skeletal muscle, stomach, and spleen, with the majority of the lysozyme mRNA being of the M type.

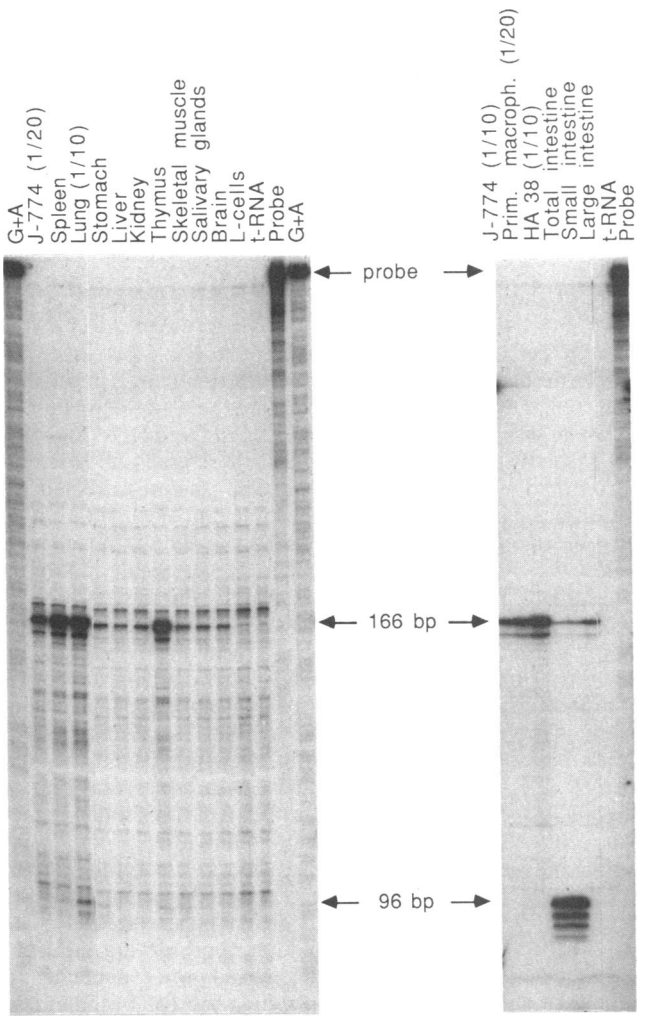

FIG. 3. RNase mapping of total RNA from mouse cell lines and tissues. The position of bands corresponding to undigested probe, protection by lysozyme M (166 nucleotides), and partial protection by lysozyme P (96 nucleotides) mRNAs are indicated. Prim. macroph., primary macrophages; G+A, purine-specific chemical cleavage reaction mixtures used as markers; Probe, undigested probe RNA; t-RNA, nonspecific protection of probe by tRNA. Ten micrograms of RNA was used in each case, except where otherwise indicated by a dilution factor. bp, Base pairs.

DISCUSSION

Comparison of the amino acid sequence derived from the mouse lysozyme M gene to partial sequences already available has revealed a number of differences between the carboxyl-terminal regions. These seem likely to reflect the necessarily preliminary nature of the tryptic peptide composition and alignment studies previously carried out, rather than actual differences between the proteins concerned. The observation that the mouse lysozyme M and chicken lysozyme genes have maintained identical intron positions throughout 300 million years of divergence, and that in this arrangement exons correlate to functional domains of the enzyme (40), is consistent with proposed mechanisms of gene evolution in which individual exons maintain a degree of autonomy (44). Indeed, such events may have given rise to the mammalian α-lactalbumin gene, the first three exons of which have marked similarity to those of the chicken (9) and mouse (Table 1) lysozyme genes. While comparison of mouse lysozyme M sequence to lysozymes of other species should now facilitate elucidation of evolutionary relationships, it will also be interesting to compare both coding and noncoding sequences to those of the mouse lysozyme P gene, the product of a relatively recent divergence (16), to study more closely the nature of the events resulting not only in different proteins, but in widely differing tissue specificities of gene expression. The possibility that mouse lysozyme P is represented among genomic clones that we found to hybridize weakly to the lysozyme M cDNA probe is currently under investigation (A. C. Wilson and G. Cortopassi, personal communication).

For the determination of tissue specificity of gene expression, dot blot hybridization detection of specific mRNA has the advantage over protein assays of being unaffected by

rates of protein secretion and accumulation, although possible effects of variations in mRNA stability in different tissues cannot be excluded. The high levels of lysozyme expression revealed in macrophages by this technique may account for the strong signals detected in some other tissues, especially lung, which has a high content of active macrophages as a barrier to infection (45), spleen, in which macrophages are present and involved in antigen presentation (46), thymus, in which macrophage/thymocyte interactions occur during T-cell maturation (47), and bone marrow, which is rich in all cells of the myeloid lineage. Intestinal lysozyme P, which may play a digestive role, is probably expressed solely by the Paneth cells (12). Despite the detection of low levels of lysozyme P in some other tissues, expression of P in primary macrophages or macrophage cell lines was not detectable.

While it is feasible that differences in the mRNA/enzyme ratios between tissues reflect variable degrees of posttranscriptional control, these ratios will also be affected by differences in the rates of secretion and accumulation of enzyme in different tissues. Hence, an accumulation of blood lysozyme is most likely to explain the extremely high concentration of enzyme in kidney, which contains only background amounts of specific mRNA. Markedly high levels of lysozyme in the spleen may also be a result of blood lysozyme accumulation, although the spleen itself contains moderate amounts of lysozyme mRNA of probable macrophage origin. A distinction between lysozyme synthesis and uptake, and determination of the sites of synthesis within tissues, should be possible at the cellular level by *in situ* hybridization of specific lysozyme nucleic acid probes and the parallel detection of protein by immunological techniques.

Our studies of lysozyme expression in macrophages and myeloid cell lines indicate a progressive and strong activation of the gene throughout macrophage differentiation. The transfection of DNA into cell lines representing different stages of myeloid development should now enable us to examine in detail elements controlling the establishment of tissue-specific gene expression in macrophages.

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