Lysozyme is an important component of innate immunity against common pathogens at mucosal surfaces. We previously cloned and characterized the bovine lysozyme 5A (lys5A) promoter with the purpose of determining cis- and trans-acting elements controlling airway epithelial cell-specific expression. We found that such expression is controlled by protein binding to an ETS consensus sequence located approximately at −46 to −40 bp from the transcription start site. The identity of the ETS-related protein responsible for gene transactivation was unknown. In this study, we screened six ETS-related proteins by transient transfection into epithelial cells and fibroblasts. Results showed that among these factors, the myeloid Elf-1-like factor (MEF) was the most potent. Gel shift analysis of epithelial cell nuclear extracts using a lys5A probe including the ETS-binding site (−50/−31) yielded a single band with retarded mobility. This band was supershifted by an antibody directed against MEF. Supporting the possibility that MEF is responsible for functional transactivation of lysozyme in epithelial cells, we found that antisense MEF mRNA decreased lys5A promoter activity and that MEF overexpression in stably transfected cells increased lysozyme mRNA and protein expression. We conclude that MEF is required for epithelial cell transactivation of lysozyme.

Lysozyme, also known as muramidase, is an enzyme catalyzing the hydrolysis of β1–4-glycosidic bonds between N-acetylmuraminic and N-acetyl-d-glucosamine, constituents of the cell walls of most bacteria. Its antibacterial properties render it an important participant in host defense at mucosal surfaces (1–3). Recent work has shown that inhibition of cationic antimicrobial proteins such as lysozyme predisposes the airway epithelium to infection (4).

At mucosal surfaces, lysozyme expression is confined to specialized epithelial cells, including the serous gland cell of the respiratory epithelium (5, 6) and the Paneth’s cell of the gastrointestinal epithelium. In hematopoietic cells, lysozyme expression is confined to the macrophage and granulocyte lineages (7). Mechanisms responsible for cell-specific expression are poorly understood. One feature that appears to be critical for expression in both cell types, however, is transactivation by ETS (E26 transforming-specific) family proteins (8). ETS proteins were initially described in the E26 avian leukemia virus (9–11), and they have now been identified in many species from Drosophila to man (12). Many myeloid-specific genes require ETS protein binding to DNA (e.g. CD4 and macrophage colony-stimulating factor) (13, 14). That ETS proteins play important roles in myeloid cell development was demonstrated by experiments showing that the targeted disruption of the PU.1 or ETS-1 gene has profound effects on development (15, 16).

Previously, we showed that epithelial cell-specific lysozyme expression required an intact ETS-binding site in the proximal promoter of the bovine lysozyme gene 5A (17). This was consistent with results indicating a requirement for binding of the ETS family transcription factor PU.1 to mediate lysozyme transcription in chicken macrophages (18). Although the identity of the lysozyme-associated ETS protein in epithelial cells was unknown, it was clearly not PU.1 since PU.1 is not expressed in epithelial cells (19).

In the studies reported here, we examined the role of various ETS family members in the control of lysozyme transcription in epithelial cells. Our results indicate that the myeloid Elf-1-like factor (MEF) is necessary for lysozyme expression in epithelial cells and is sufficient to mediate lysozyme transcription in fibroblasts.

**Experimental Procedures**

**Cell Culture and Transfections—**Cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO2 and 95% air atmosphere.

Transient transfections were performed with Transfectam (Promega) according to the manufacturer’s recommendations. Specifically, 10 μl of Transfectam reagent and 4 μg of total DNA in Dulbecco’s modified Eagle’s medium were incubated for 10 min before the mixture was applied to subconfluent cells on six-well plates. Cotransfection of various plasmids was performed with 2 μg of reporter and 2 μg of each effector. Empty vector (pCB6) was added where necessary to ensure a constant amount of input DNA. Cotransfection with the pRL-CMV vector (10 ng in each sample), which expresses Renilla luciferase (Promega), verified that differences in firefly luciferase reporter gene expression were not due to differences in transfection efficiency. Cells were incubated for 2 h with the DNA mixtures, at which time additional medium was added. Forty-eight hours after transfection, the medium was removed, and cells were harvested. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega) and a luminometer (Lumat LB9507, EG&G Berthold). Absolute light emission generated from the luciferase enzyme reaction was determined. Relative luciferase activity is plotted and represents the -fold induction of activity generated by experimental treatments with respect to activity associated with basic vector alone. Values are shown as means ± SEM.

*This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan and by National Institutes of Health Grants R0143762 and P0124136 (to C. B.). The costs of publication of this article were deferred in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: MEF, myeloid Elf-1-like factor; PCR, polymerase chain reaction; bp, base pair(s); WT, wild-type; lys5A, lysozyme 5A; GST, glutathione S-transferase.

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**Myeloid ELF-1-like Factor Up-regulates Lysozyme Transcription in Epithelial Cells**

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To normalize expression levels of the transcription factors, we measured mRNA by Northern blotting using the transcribed sequence of pCB6 as a probe.

For generation of stably transfected clones, introduction of expression constructs into the human lung adenocarcinoma cell line A549 (RCB0098) was performed by electroporation. Approximately 1 x 10^6 cells were transfected with 100 µg of MEF cDNA in pCB6 linearized with ApaLI. Electroporations were performed using an ECM 600 apparatus (BTX Inc.) at 500 V and 1350 microfarads. Cells were then cultured on six-well plates at 1 x 10^5/well. At 50% confluency, 1 mg of G418 sulfate (Calbiochem)/ml was added. G418-resistant clones were picked 1 week later and analyzed individually or as pools of several hundred clones. Stably overexpressed cells were selected by Northern blotting.

**Plasmid Constructs**—The (−100 bp)/WT lys5A promoter (−100/+10) was prepared by PCR using, as a template, the previously described plasmid pCATBi−170/+10 (17). We used the following oligonucleotide primers: 5′-primer, GACCTCGAGGCTGACCCA; and 3′-primer, ATAAACGCGTCCTATTTTCCACAA. The PCR product was cloned into the MluI and XhoI sites of pGL2-basic (Promega), a promoterless luciferase expression plasmid.

The (−50 bp)/WT lys5A promoter (−50/+10) was also prepared by PCR using, as a template, (−100 bp)/WT and the following oligonucleotide primers: 5′-primer, CCGCTCGAGAAGAAGAAGTGAAAAGA-
TG; and 3'-primer (GL primer 2), CTTTATGTTTTTGGCTGCTCCC. It was cloned into the MluI and XhoI sites of pGGL2.

(−100 bp)E-M is the construct (−100 bp)/WT with a mutated ETS site. It was prepared using a Transformer site-directed mutagenesis kit (CLONTECH) and BLp90-sense as a mutation primer. It contains −60 to −31 bp, with a mutated ETS site indicated in lowercase boldface letters, CCAGTCATAGAAGGTGTTAAAAAGTG.

(−50 bp)E-M is the construct (−50 bp)/WT with a mutated ETS site. It was prepared using a Transformer site-directed mutagenesis kit and BLp90-sense as a mutation primer.

A full-length cDNA (1992 bp) for human MEF (20) was obtained by reverse transcription-PCR. This was done with a GeneAmp RNA PCR kit (Perkin-Elmer) and the following primers: 5'-primer, CGGGATCCCGGATCCGGATCCGGATC; and 3'-primer, GGAATTCCTGTAATGCTCATGGGGCCGGTCCATG. The PCR product was cloned into the pCR2.1 vector using the Original TA cloning kit (Invitrogen). After digestion of the sequence, it was cloned into the BglII/XhoI site of pCB6 downstream of the cytomegalovirus promoter. All constructs were verified by DNA sequencing.

For generation of antisense mRNAs of MEF and ESE-1, the cDNA in pCB6 was cut with EcoRI and religated to get a reverse orientation of MEF and ESE-1. The resulting plasmid was sequenced to verify insert orientation.

**Electrophoretic Mobility Shift Assay—** A549 cells (0.5–1 × 10⁶) were collected, washed with 10 mL of Tris-buffered saline, and pelleted by centrifugation at 1500 × g for 5 min. The pellet was resuspended in 1 mL of Tris-buffered saline, transferred into an Eppendorf tube, and pelleted again by spinning for 15 s in a microcentrifuge. Tris-buffered saline was removed, and the cell pellet was resuspended in 400 μL of cold buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride by gentle pipetting. The cells were allowed to swell on ice for 15 min, after which 25 μL of a 10% solution of Nonidet P-40 (Nakarai) was added, and the tube was vigorously vortexed for 10 s. The homogenate was centrifuged for 30 s in a microcentrifuge. The nuclear pellet was resuspended in 50 μL of ice-cold buffer containing 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, and the tube was vigorously rocked at 4 °C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microcentrifuge at 4 °C, and the supernatant (−55 μL) was frozen in aliquots at −80 °C.

The following complementary oligonucleotides were synthesized and used in electrophoretic mobility shift assays: wild-type oligonucleotide (which contains lys5A promoter sequences from −50 to −31 bp), AAAGAAGGAACTGAAAAGAGT; and ETS mutant (which contains −50 to −31 bp, with a mutated ETS site indicated in lowercase boldface letters), AAAGAAGGAACTGAAAAGAGT. The wild-type oligonucleotide was labeled with [γ⁻³²P]ATP and incubated with 2 μg of double-stranded poly(dI-dC) in the absence (lanes 1 and 2) and presence (lanes 3–7) of 1 μg of nuclear protein. The following unlabeled double-stranded competitor oligonucleotides were added at a 100-fold molar excess over the probe oligonucleotide: wild-type competitor (WT; lane 4) and mutated competitor (MT; lane 5). Nonimmune antibody (N; lane 6) or anti-MEF antibody (MEF; lane 7) was added to the reaction mixture.
A screen of ETS transcription factors (Elf-1, Ets-1, Ets-2, PEA3, ESE-1, and MEF) revealed that despite the presence of an ETS consensus binding sequence on the lys5A promoter, most ETS proteins were unable to activate lysozyme transcription. The only ETS proteins active in this regard were MEF and ESE-1 (Fig. 1). MEF stimulated transcription more strongly than did ESE-1 in lung epithelial cells (A549), colon carcinoma cells (Caco-2), and skin fibroblasts (NIH3T3) (Fig. 1). That this occurred through MEF binding to the ETS consensus sequence was indicated by the fact that activation was inhibited when the ETS consensus sequence in the lysozyme regulatory region was changed from 5'-GGAA-3' to 5'-GGTC-3'. The reason for the weak inhibition by the mutation in Caco-2 cells is unclear.

To determine whether or not MEF was endogenously present in the nuclei of epithelial cells and could thereby mediate endogenous lysozyme expression, we performed gel shift and supershift assays. As a probe, we used the radiolabeled lys5A promoter −50/−31 oligonucleotide. We observed a single band with retarded mobility after probe incubation with A549 cell nuclear extract (Fig. 2). That the band represented a specific DNA/protein interaction was evident from experiments showing competitive inhibition of band formation using excess unlabeled wild-type probe, but not the probe mutated at the ETS consensus binding site. That MEF was present in the complex was indicated by assays showing that anti-MEF antibody, but not preimmune serum or antibodies directed against human Ets-1, Ets-2, PU.1, or PEA3 (Santa Cruz Biotechnology) (data not shown), caused a supershift of the DNA-protein complex.

To determine whether or not endogenous MEF is required for basal activity of the lys5A promoter, we produced antisense MEF mRNA in A549 cells using expression plasmid pCB6 with MEF cDNA in reverse orientation. The presence of antisense mRNA was associated with an 80% inhibition of luciferase activity for lys5A−(−50/+10) and 30% inhibition for lys5A−(−100/+10) (Fig. 3). In addition, the presence of antisense ESE-1 mRNA with antisense MEF mRNA strongly inhibited luciferase activity for lys5A−(−100/+10). This construct contains enhancer-like activity in the region at −94 to −66 bp (17), although the precise DNA sequence and cognate binding protein remain to be identified.

We obtained further evidence for the role of MEF in lysozyme transcription by stably overexpressing MEF in A549 cells. The stable transfectants are referred to as line 71. MEF overexpression strongly stimulated transiently transfected lys5A promoter activity if cells were transfected with wild-type constructs, but not with constructs mutated at the MEF DNA-binding site (Fig. 4A). In addition, MEF overexpression upregulated endogenous lysozyme mRNA and protein expression, but not mRNA encoding the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (Fig. 4, B and C).

**DISCUSSION**

The studies reported here indicate that the ETS protein MEF is present in lung epithelial cells and is required for lysozyme transcription in these cells. This is the first information regard-
MEF Up-regulates Lysozyme Transcription in Epithelial Cells

...ing requirements for lysozyme expression in epithelial cells, as previous studies have focused on macrophages. Although lysozyme transcription in macrophages and epithelial cells is similar in requiring ETS protein transcription factors, the two cell types differ in their specific use of ETS proteins. Thus, whereas macrophages activate lysozyme transcription via PU.1, our data indicate that epithelial cells use, at least in part, the ETS family member MEF.

We found that in epithelial cells, MEF up-regulated not only the activity of a transiently transfected lysisA promoter, but also the transcription of the endogenous lysozyme gene and protein. Moreover, antisense experiments showed that inhibition of endogenous MEF in epithelial cells attenuated the baseline level of lysozyme expression. Taken together, these results indicate that MEF is required for base-line expression of lysozyme in epithelial cells.

The finding that antisense MEF constructs did not completely block lysozyme expression suggests that other factors are also involved in A549 cells. A strong candidate is the ETS family member ESE-1/ERT/ESX/ELF-3 since antisense ESE-1 constructs also partly inhibited promoter activity, since it is known to exist in epithelial cells, and since ESE-1 overexpression was seen to up-regulate lysisA promoter activity in cotransfection experiments (Fig. 1). With respect to lysozyme up-regulation, we are aware of the existence of a second element (−100/−51) that is well conserved in the human lysozyme gene. Although the enhancer remains to be identified, MEF may require interaction with the enhancer-binding protein to activate lysozyme expression.

Our results showing a requirement for ETS family proteins in the expression of lysozyme in epithelial cells are consistent with previous data showing that the ETS protein PU.1 is required for lysozyme expression in chicken macrophages (18). The PU.1-binding site is present in an enhancer located 2.7 kilobases upstream of the transcription start site of chicken lysozyme. Mutation of this site abolishes enhancer activity in macrophages (18). Unclear at this point is the issue of whether epithelial cells in chicken, like those in mammals, use MEF and/or ESE-1 to activate the response element activated by PU.1 in macrophages.

A subtle contrast with the findings reported here are results regarding the mouse M lysozyme gene. This gene, although also regulated by ETS transcription factors, is flanked by ETS consensus sequences lying 3′, rather than 5′, to the gene coding region. The 3′-region containing ETS consensus sequence is co-extensive with DNase I hypersensitivity sites that under demethylation in lysozyme-expressing macrophages (27) and is therefore likely to be functionally important.

A major focus of our laboratory’s research is the differentiation and function of respiratory tract epithelial cells. In this regard, it is interesting that the 5′-flanking regions of several genes selectively expressed in respiratory tract serous cells contain ETS protein-binding sites. Specifically, the motif is present flanking genes encoding proline-rich proteins (28), lactoferrin (29), the cystic fibrosis transmembrane conductance regulator (30), and secretory leukocyte protease inhibitor (31). The presence of shared transcription factor-binding sites may denote the presence of a regulatory cascade that controls serous cell differentiation in a manner similar to that already described for more extensively studied tissues such as skeletal muscle (32).

In summary, these studies provide the first information identifying transcription factors controlling lysozyme expression in epithelial cells. As bacteria become progressively more resistant to exogenously applied antibiotics, information regarding mechanisms controlling innate immunity, such as that provided by epithelial lysozyme, may offer important new therapeutic approaches.

Acknowledgments—We thank Dr. T. A. Libermann for kindly providing the ESE-1 cDNA, Dr. D. K. Watson for Ets-1 and Ets-2 cDNAs, and Dr. J. A. Hassel for PEAS.

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