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Transcriptional Regulation of the Lysozyme Gene in Airway Gland Serous Cells

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Abstract Lysozyme is expressed in serous, but not mucous, cells of the tracheobronchial glands and thereby constitutes a marker of the serous cell lineage in these glands. To identify DNA regulatory elements and transcription factors mediating the commitment of progenitor cells to the serous cell lineage, we have characterized the regulatory activity and DNA-protein interactions of the 5′-flanking region of the bovine lysozyme gene *lys 5a*. Results obtained from these studies indicate that although approximately 94 bp of 5′ flanking DNA are necessary for high level expression in transient transfection assays, an evolutionarily conserved promoter within 66 bp of the transcription start site is sufficient to confer serous cell-specific expression. Farther upstream, within 6.1 kb of the 5′ flanking region, are 4 silencers. Analysis of the serous cell-specific lysozyme promoter by electrophoretic mobility shift assay (EMSA) revealed the presence of binding sites for 3 serous cell nuclear proteins, designated LSF1, LSF2 and LSF3. Binding of LSF2 and LSF3 was localized to a 20-mer subdomain (−50/−30) of the cell-specific promoter using binding competition assays. More accurate identification of the protein binding site(s) was achieved through the use of mutagenesis, which implicated the motif 5′AAGGAAT 3′ (−46/−40) in both protein binding and serous cell-specific transcriptional activity. This motif has previously been identified as a binding site for ets protein transcription factors, suggesting that serous cell-specific regulation of *lys 5a* transcription is partly controlled by the binding of ets-like protein(s) to the motif 5′AGGAAGT3′.

Key words: lysozyme, gene regulation, cell differentiation, serous cells, gland cells

INTRODUCTION

During early tracheobronchial development, the epithelium lining the air space is composed of multipotential progenitor cells [Jeffery and Reid, 1977]. These give rise to ciliated, goblet, and basal cells, as well as to submucosal glands consisting of serous and mucous cells. While generally present in fixed proportions throughout adult life, these cell types vary in relative abundance in human airway diseases such as chronic bronchitis, in association with considerable morbidity [Takizawa and Thurlbeck, 1971; Takizawa and Thurlbeck, 1971b]. With the aim of understanding these cells' differentiation programs both in development and in disease, we are attempting to identify some of the molecular mechanisms responsible for their cell-specific gene expression.

A striking example of specificity is seen in the expression of lysozyme by serous gland cells in tracheobronchial tissue. Lysozyme (also known as muramidase [EC 3.2.1.17]), is an enzyme catalyzing the hydrolysis of β (1–4) glycosidic bonds between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine, constituents of the cell walls of most bacteria. Its antibacterial properties render it an important participant in mucosal defense at body surfaces and in leukocytes [Klockars and Osserman, 1974; Klockars and Reitamo, 1975; Mason and Taylor, 1975]. Our previous work [Dohrman et al., 1994] has indicated that the selective expression of lysozyme in the serous gland cell [Bowes and Corrin, 1977] is attributable to regulatory mechanisms controlling steady state mRNA.

A common mechanism by which cells control steady state levels of specific mRNAs is through protein-DNA interactions occurring in the 5′

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flanking region of a gene. With respect to lysozyme, this type of interaction has been most extensively analyzed in the chicken. In the oviduct of that species, lysozyme expression requires glucocorticoid regulation, whereas myeloid cells express lysozyme constitutively. Although glucocorticoid receptor binding sites have been identified both in the gene promoter (−39/−74 and −161/−208) [Matthias et al., 1982; Renkawitz et al., 1984] and farther upstream [Hecht et al., 1988], expression studies have shown that only the promoter elements are required for glucocorticoid-regulated transcriptional activity [Renkawitz et al., 1984]. Lysozyme expression in myeloid cells has been shown to be regulated by positive elements at −6.1 and −2.7 kb and between −208 and −66 bp [Theisen et al., 1986; Steiner et al., 1987]. Negative elements have been detected at −2.4, −1.0, and between positions −260 and −207 [Baniahmad et al., 1987; Steiner et al., 1987].

Although mammalian lysozyme genes have been studied much less extensively, investigation of the mouse M-lysozyme gene has revealed the presence of a cluster of cell- and stage-specific DNase I hypersensitivity sites in the 3′ region of the gene. This region contains an enhancer that shows no cell- or stage-specificity in transient transfection assays [Möllers et al., 1992] but during the course of in vivo myeloid differentiation is specifically activated by demethylation [Klages et al., 1992].

To obtain information concerning transcriptional regulation of lysozyme in mammalian tracheobronchial serous cells, we have performed transient transfection assays in serous gland cells isolated from the cow trachea [Finkbeiner et al., 1986]. Because at least 10 lysozyme genes exist in the cow [Irwin et al., 1989], identification of *lys 5a* as the principal lysozyme gene expressed by cow serous cells was a necessary first step [Takeuchi et al., 1993]. In the present study, we sought to identify regions of the 5′ flanking region of this gene that regulate its expression, with particular attention to those regions conferring serous cell-specificity. Our analysis suggests that serous cell-specific expression is partly mediated by ets-related nuclear proteins binding to the motif 5′AGGAAGT 3′. This work has appeared in abstract form [Kai and Basbaum, 1994].

MATERIALS AND METHODS

Genomic cloning and sequencing of the *lys 5a* gene flanking region. A bovine genomic library packaged in phage EMBL-3 (Clontech, Palo Alto, CA) was screened using the synthetic oligonucleotide primer KT17, 5′-TTCCCCTAAAGTTATCCATTCCAAATCTT-3′, an anti-sense fragment corresponding to an upstream region of a cDNA specific for the *lys 5a* gene [Takeuchi et al., 1993]. This oligonucleotide was labeled with ^{32}P using T4 DNA kinase (specific activity 6×10^8 cpm/mg) and used to screen 6×10^6 plaques. Duplicate nitrocellulose filter plaque lifts were hybridized in 1.2 × SSC, 2 × Denhardt's, 0.25% sodium dodecyl sulfate and 100 mg/ml salmon sperm DNA at 45°C. After hybridization, the filters were washed at 40°C in 2 × SSC and 0.05% SDS for 1 hr, and then washed at 50°C in the same solution for 10 min. Positive clones were purified through additional cycles of plaque screening [Sambrook et al., 1989]. Portions of genomic clones containing *lys 5a* sequence as determined by Southern blot were subcloned into the BamHI site of the vector pBluescript II (Stratagene, La Jolla, CA). The clone insert containing the 1400 bp immediately upstream of the transcription start site was completely sequenced in both directions using the Sequenase dideoxy chain termination sequencing kit (US Biochemical Corp.)

Mapping the transcription start site of lys 5a in tracheal gland serous cells—primer extension. The synthetic oligonucleotide primer KT17 (described above) was 5′-end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled primer was hybridized to 50 μg of total RNA from bovine trachea and extended by reverse transcriptase under conditions of cDNA synthesis [Sambrook et al., 1989]. The reaction mixture was ethanol-precipitated, redissolved in loading buffer and analyzed on a 6% polyacrylamide, 7 M urea sequencing gel.

S1 nuclease protection analysis. The primer KT17 described above was used in a procedure similar to that described in [Ausubel et al., 1987]. As a template for DNA synthesis, a 7 kb Bam HI restriction fragment of genomic clone 163a was cloned into pBluescript II SK-. Ten μg denatured template DNA was annealed to 5 pmol 5′-end labeled primer. Unlabeled dNTPs were added to a final concentration of 400 μM and DNA synthesis was carried out by incubation with 5 U of the Klenow fragment of *E. coli* DNA polymerase I. The DNA was cleaved with EcoRI and applied to a 4% denaturing polyacrylamide gel to purify the single stranded DNA probe. Purified probe (3×10^4 cpm) and 5

μg poly A^+ RNA were denatured at 85°C for 10 min and hybridized overnight at 48°C [Ausubel et al., 1987]. Samples were digested with 700 units S1 nuclease (BRL) in 200 μl S1 buffer containing 300 mM NaCl, 70 mM sodium acetate pH 4.7, 1.5 mM $ZnSO_4$, and 6 μg denatured salmon sperm DNA at 30°C for 1 h. After ethanol precipitation and redissolving in loading buffer, protected fragments were analyzed on a 6% denaturing polyacrylamide gel.

Plasmid constructs. To identify functional promoter elements, we cloned in pCAT Basic vector (pCATB, Promega) a panel of plasmid constructs containing sequential deletions of the *lys 5a* 5′ flanking sequence fused to the reporter gene chloramphenicol acetyltransferase (CAT). pCATB (−170/+10) contained a fragment of the *lys 5a* 5′ flanking region generated by PCR using as 5′ primer KT30: GATTAAAGCTTAGGAACATCAGAATG and as 3′ primer KT29: AGAAGGTCGACCTCCAGGCTGACCC. The PCR product digested with Sal I and Hind III was ligated into the multiple cloning site of pCATB. pCATB (−6100/+10) was made from CATB (−170/+10) and the 6.1 kb Hind III-Sac I fragment of genomic clone 163a. Other plasmids were constructed by standard techniques from CATB (−170/+10) and CATB (−6100/+10). To identify enhancer and silencer elements, we used pCAT®-Promoter Vector (Promega). Sequences of CAT constructs were confirmed using the Sequenase dideoxy chain termination sequencing kit (US Biochemical Corp.). Mutation-containing constructs were made using Luciferase Basic Vector and mutated oligonucleotides.

Cell culture. Bovine tracheal gland serous cells and fibroblasts were isolated separately by previously described methods [Finkbeiner et al., 1986]. Each cell type was cultured in Ham's F12:Dulbecco's MEM H21 (1:1) with 10% fetal bovine serum and antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin) in plastic culture flasks or plates.

DNA transfection and CAT assay. Plasmids were banded in CsCl prior to transfection. Transfections were performed as follows. Approximately 70% confluent cells in 6-well plastic plates were incubated for 5 h with 10 μl Lipofectin® (Gibco BRL), 5 μg of CAT constructs, and 2 μg of pSV-β-Galactosidase Control Vector® (Promega, an internal control for transfection efficiency, which contains the SV40 promoter and enhancer fused to a β-galactosidase reporter gene). After a change of medium, cells were incubated for 43 h before harvesting. The cells were collected from the tissue culture plates, and cell extracts were made by freezing and thawing (4–5 x) in dry ice/ethanol and a 37°C water bath. A portion of the cell extract was used for β-galactosidase assay, performed using Galacto-Light® (TROPIX). The remaining cell extract was heated for 10 min at 65°C and used for determination of CAT activity. The CAT assay reaction mixture included 100 μl of cell extract, 2 μl of ^{14}C-chloramphenicol (56.8 mCi/mmol, DuPont, NEN), 5 μl of n-butyryl coenzyme A (5 mg/ml in 1 M Tris-HCl, pH 7.5, Sigma), and the reaction buffer (1 M Tris-HCl, pH 7.5) in a total volume of 150 μl. A 16 h incubation at 37°C was used for all reactions based on pilot experiments demonstrating linearity of enzyme activity under those conditions. The reaction was terminated by extraction with 300 μl of xylene. The xylene layer was transferred to a fresh tube and back-extracted 3 times with 100 μl of reaction buffer to remove any [^{14}C]chloramphenicol or degraded products. The xylene layer was then transferred to a glass scintillation vial for counting. The level of CAT activity per unit of plasmid DNA introduced by transfection (based on β-galactosidase activity levels) was expressed for each experiment. Values shown are averages derived from two representative experiments involving two independent transfections in which each construct was tested in triplicate. The experiments yielded similar results, yielding standard deviations corresponding to less than 20% of the mean.

Electrophoretic mobility shift assays. Nuclear extracts from bovine submucosal gland serous cells and bovine tracheal fibroblasts were prepared according to [Dignam et al., 1983]. The protein concentration of the cell extract was determined using a BCA protein assay kit (Pharmacia LKB Biotechnology Inc.). SacI—SalI fragments (76 bp) of pCATB (−170/+10) were used in EMSA. The fragments were labeled with α-^{32}P using the Klenow fragment of DNA polymerase I. Binding assays were done by preincubating 5–10 μg of crude nuclear extract, 2 μg of poly(dI-dC) (Pharmacia, Piscataway, NJ), and bovine serum albumin to 300 μg/ml, with or without the relevant unlabeled DNA fragment or a double stranded oligonucleotide as a competitor, in 20 μl 12 mM HEPES, pH 7.9, 12 mM KCl, 0.6 mM $MgCl_2$, 1.2 mM DTT, 75 mM NaCl, and 10.2% glycerol for 5 min at room temperature. The labeled DNA fragment was then added to the incubation mixture, which was kept at 30°C for 15 min. Reaction products were analyzed by

electrophoresis on a 4% polyacrylamide gel in high ionic strength buffer according to [Ausubel et al., 1987] followed by autoradiography at −70°C.

Mutagenesis of the 5a lysozyme promoter by PCR. Oligonucleotides M (corresponding to sequence −50/−30) and M1–M4 (containing sequential dinucleotide mutations of −50/−30) were used as upstream primers. The downstream primer was a 15-mer oligonucleotide derived from pCATBasic vector. Template DNA was pCATBasic (−66/+10). The thermal cycle reactions were 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min (30 cycles). The PCR products were subcloned into TA cloning vector (Invitrogen, San Diego, CA) and digested with Sac I and Sal I, and then ligated into pLUCB. Mutated sequences were confirmed by sequencing.

RESULTS

A Genomic Clone containing the 5′-flanking region of the 5a lysozyme gene. Genomic clone 163a was isolated by screening a genomic library (6×10^6 clones) with oligonucleotide probe KT17, corresponding to a portion of the *lys 5a* exon I [Takeuchi et al., 1993]. Insert size was approximately 12 kb. A restriction map of the gene fragment is shown in Figure 1. Southern blots using exon-specific oligonucleotide probes revealed that exons 1 and 2, but not 3 were present in 163a. Subsequent sequencing revealed that the nucleotide sequence of the two exons present in the genomic clone was identical to that in cDNA clone 5a reported previously [Takeuchi et al., 1993].

Characterization of the 5′ end of the lys 5a mRNA. To identify the 5′ end of the mRNA synthesized in tracheobronchial serous gland cells, we performed primer extension and S1 nuclease mapping (Fig. 2). Two bands (at 28 and 25 nucleotides upstream of the translation start site) were detected by primer extension. A major band at 26 nucleotides upstream of the translation start site was detected by S1 nuclease mapping. We defined the latter as a major transcription start site for the 5a lysozyme gene in tracheobronchial serous cells.

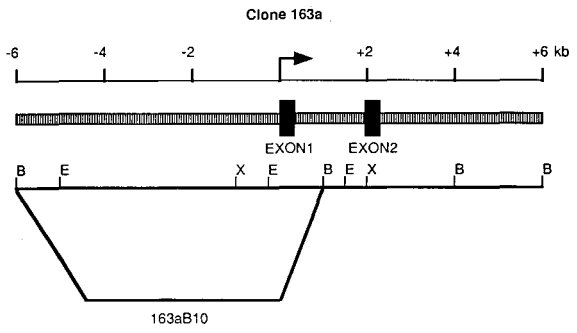

Fig. 1. Schematic representation of a genomic clone (163a) of the bovine 5a lysozyme gene (*lys 5a*). A restriction map of the clone is shown. Restriction sites are as follows: E, EcoRI; B, BamHI; and X, XbaI. 163aB10 is a subclone from which the constructs used in the transient transfection assays were made.

Nucleotide sequence of the 5′-flanking region of the 5a lysozyme gene. Partial sequencing of subclone 163aB10 (Fig. 1) provided the sequence of the 5′-flanking region extending to bp −1395. Most of this sequence, and that of part of exon 1 are shown in Figure 3. Computer analysis revealed the presence of potential transcriptional regulatory elements including a TATA-like element with the sequence TAAAAA, a PEA3 site, two CCAAT/enhancer-binding protein (C/EBP) sites (TGTGGATTTGC), a palindromic glucocorticoid response element (TGTTCTNNNAGAACA) and two AP1 sites (TGA[G/C]T[C/A]A).

Comparative analysis of promoter regions of cow, human, mouse, and chicken lysozyme genes. As shown in Figure 4, the first 100 bp upstream of the transcription start site of *lys 5a* are highly similar to corresponding regions of the cow 2 (stomach) lysozyme gene (83% identity), [Irwin et al., 1993], and human lysozyme gene (78% identity) [Peters et al., 1989a]. In addition, they are moderately similar to corresponding regions of the mouse M [Cross et al., 1988], chicken [Grez et al., 1981], and rat [Yeh et al., 1993] lysozyme genes (63%, 58%, and 43% identity, respectively).

Functional mapping of the cow lys 5a promoter and regulatory regions. To identify DNA sequences involved in regulation of *lys 5a* expression, we examined a series of plasmids containing variable length fragments of the lysozyme 5′ flanking sequence fused to a CAT expression unit. All DNA fragments extended to a downstream position of +10. Plasmids were transfected into both bovine tracheal gland serous cells and bovine tracheal fibroblasts. The promoter activity of each construct is shown in Figure 5. For simplicity, only the 5′ end is indicated: e.g., −66/+10 is "−66." Transfection of CATB (−66/+10) into serous cells yielded a level of CAT expression equivalent to approximately 10% of that obtained using the CAT-Control vector driven by the SV 40 promoter. The same construct transfected into fibroblasts

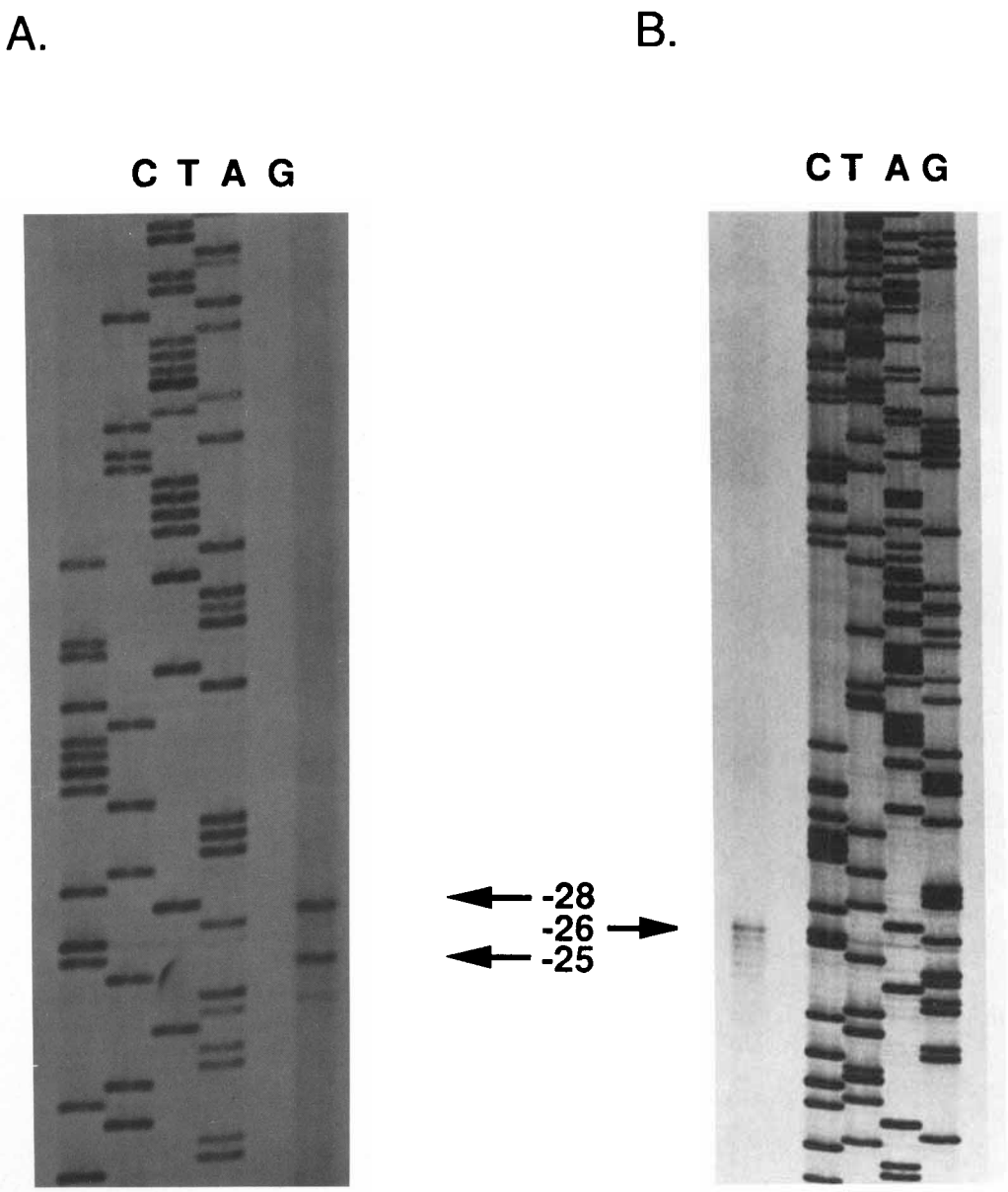

Fig. 2. Primer extension and S1 nuclease mapping analysis of the transcription initiation site of *lys 5a*. The products of a primer extension assay **(A)** and an S1 nuclease protection assay **(B)** are shown together with the DNA sequence of the promoter region of the cow lysozyme gene *lys 5a* (5′ to 3′ proceeding from top to bottom). A: *Arrows* indicate the major sites of mRNA initiation in tracheal tissue (at 28 and 25 nucleotides upstream of the translation start site). B: *Arrow* indicates a single band protected by tracheal gland cell RNA in an S1 nuclease mapping experiment. The band indicates transcription initiation at 26 nucleotides upstream of the translation start site. A control sequence reaction for 163a using KT17 as a primer is shown in lanes *C, T, A,* and *G*.

yielded less than 1% of CAT Control activity (ten-fold less than in serous cells), suggesting that −66/+10 contains a serous cell-specific element. Cloning in the reverse orientation (Fig. 5, −66R) led to markedly reduced activity.

Incorporation of additional upstream sequence to position −94 resulted in a five-fold increase of transcriptional activity in serous cells and a nine-fold increase in fibroblasts relative to the activity of CATB (−66/+10). This suggests the presence of one or more enhancer-like activities in the region −94 to −66. To investigate this, we cloned the fragment containing this region into a plasmid in which CAT transcription was under the control of the SV40 promoter. As shown in Figure 6, the putative enhancer produced a 1.5-

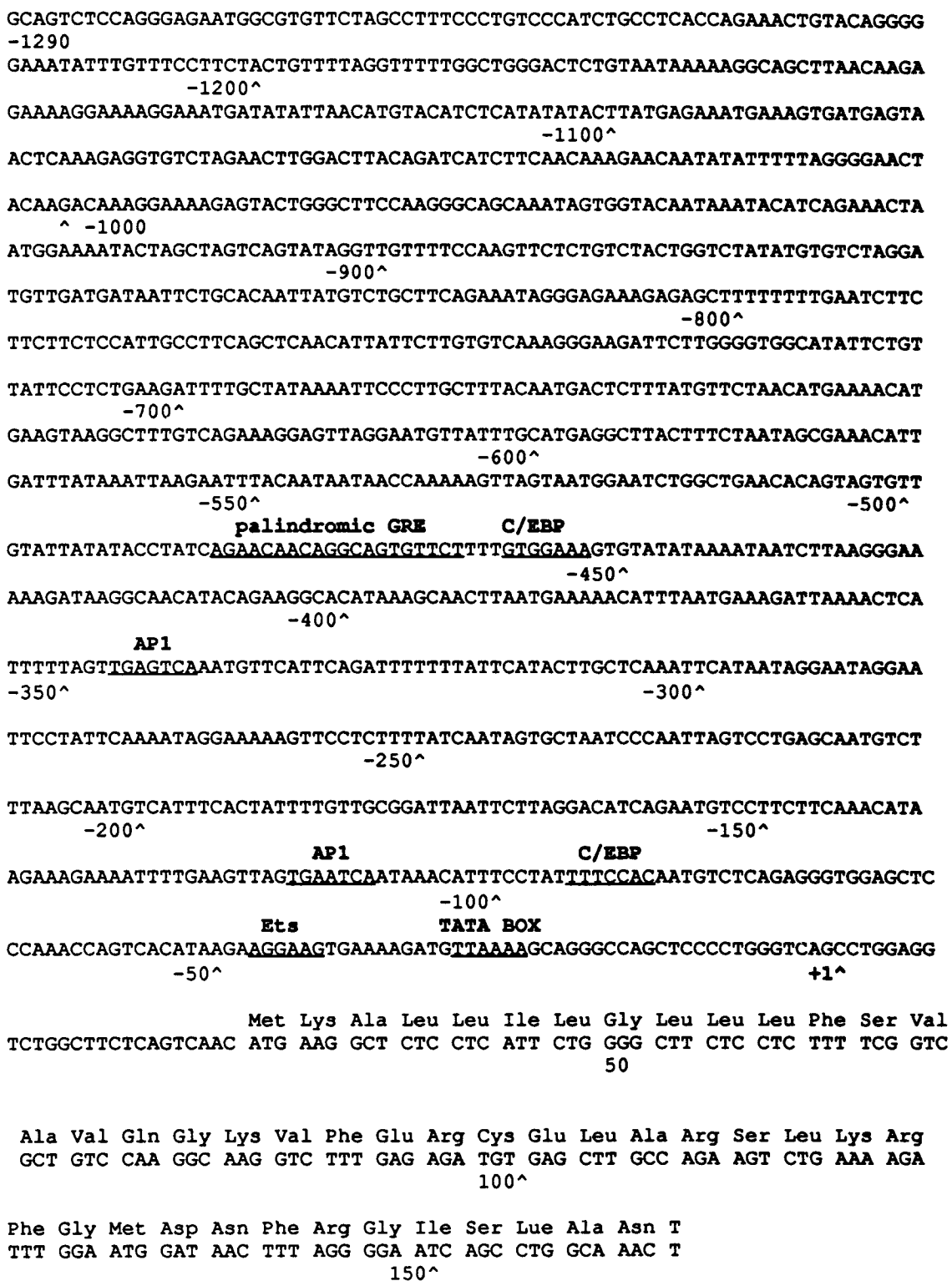

Fig. 3. DNA sequence of the 5′-flanking region of the 5a lysozyme gene and part of exon 1. Nucleotide numbering is relative to the transcription initiation site, which is designated +1. TATA box and transcription factor consensus sequences are underlined.

Fig. 4. Sequence comparison of the 5′-flanking regions of several lysozyme genes. Gaps (indicated by *dashes*) were introduced to optimize similarity. Nucleotide identities are indicated by *bars* (|). Sequences for comparison are from cow stomach 2 [Irwin et al., 1993], human [Peters et al., 1989b], mouse [Cross et al., 1988], chicken [Grez et al., 1981], and rat [Yeh et al., 1993] lysozyme gene 5′ flanking regions.

fold increase in CAT activity relative to that of CATP alone in both serous cells and fibroblasts (Fig. 6). This relatively weak effect suggests an inverse relationship between transcriptional activity and potential amplification by this enhancer.

Incorporation of additional upstream sequence between residues −94 and −170 led to decreases in CAT activity in both cell types (Fig. 5) indicating the presence of one or more silencer-like activities in this region. At least three additional silencer-like activities were present farther upstream (between −2500 and −1292, −4600 and −2500, and −6100 and −4600). Cloning and analyzing these sequences in CATP demonstrated their ability to inhibit expression of a heterologous promoter (Fig. 6) in a position- and orientation-independent manner signifying that they are true silencers. The silencers inhibited expression to a similar degree in serous cells and fibroblasts.

Electrophoretic mobility shift analysis (EMSA) of the serous cell-specific cis-element in the 5a lysozyme gene. Our findings implicating the sequence −66/+10 in serous cell-specific control of *lys 5a* transcription (see above) led us to seek binding sites for serous cell nuclear proteins in this region. The radiolabeled Sac I/Sal I restriction fragment −66/+10 served as a probe for EMSA analysis and was incubated with nuclear proteins from serous cells and fibroblasts (Fig. 7). Probe mobility was unaffected by incubation with fibroblast nuclear extract. In contrast, three bands (B1, B2, and B3) with retarded mobility were present after probe incubation with the serous cell nuclear extract. That these bands represented DNA-protein complexes was indicated by competitive inhibition with excess cold probe (not shown). To begin to define the protein binding sites, we performed competitive inhibition experiments with oligonucleotides corresponding to subdomains of the −66/+10 probe sequence (oligonucleotide −66/−50, oligonucleotide −50/−31, and oligonucleotide −31/−1). Whereas probe incubation with oligonucleotides −66/−50 and −31/−1 did not reduce formation of the DNA-protein complexes, oligonucleotide −50/−31 effectively inhibited the formation of B2 and B3, indicating that this sequence participated in protein binding. We refer to the nuclear factors responsible for the formation of B1, B2, and B3 as lysozyme serous cell factors (LSF) 1, 2, and 3.

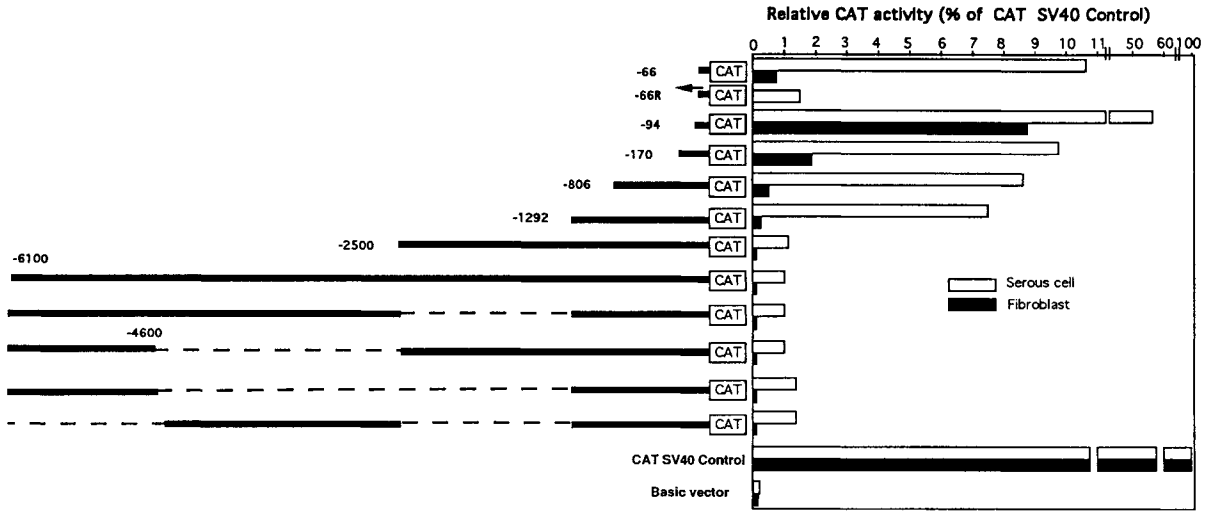

Fig. 5. Functional analysis of deletion mutants of cow lysozyme gene 5′ flanking region. Levels of chloramphenicol acetyltransferase (CAT) expression by fusion constructs of the *lys 5a* 5′-flanking region and a CAT reporter gene are shown relative to the expression of the positive control pCAT SV40. The 3′ end of each experimental construct is at +10. For simplicity, only the position of the 5′ end is indicated. R indicates reverse orientation. Values shown are averages derived from two representative experiments involving two independent transfections in which each construct was tested in triplicate. The experiments yielded similar results, yielding standard deviations corresponding to less than 20% of the mean.

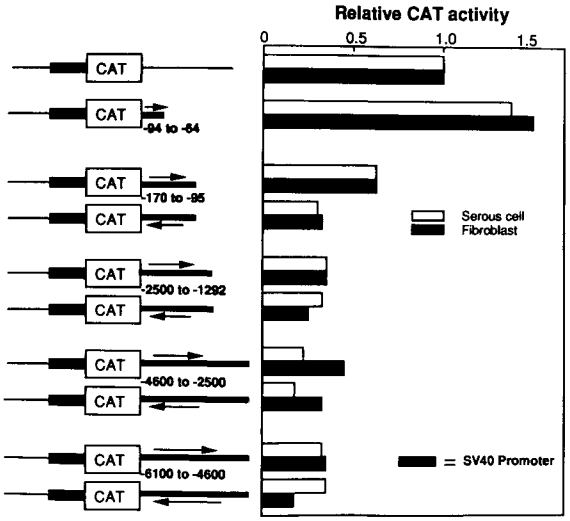

Fig. 6. Characterization of enhancer and silencer elements of the 5a lysozyme 5′-flanking region in serous cells and fibroblasts. Enhancer region (−94 to −64) and silencer regions (−170 to −95, −2500 to −1287, −4600 to −2500, −6100 to −4600) were subcloned in the pCAT SV40 Promoter vector. Levels of CAT expression driven by lysozyme gene regulatory elements are shown relative to those produced by the pCAT SV40 Promoter alone. *Arrows* indicate fragment orientation in the vector. Values are means obtained as for Figure 5.

To more accurately define the protein binding sites, we examined the effects of sequential 5′ dinucleotide mutations (yielding oligonucleotides M1-M4, Fig. 8) on oligonucleotide −50/−31-dependent inhibition. Whereas three of the four mutations had no effect, mutation 2 (M2, 5′-AAGAAGG*tc*GTGAAAAGATG-3′) effectively abolished inhibition. This suggested that the sequence containing M2 is critical for serous cell protein binding.

To determine the effects of mutation on the promoter activity of pCATB (−66/+10), we tested mutations M1–M4 in transient transfection assays. Whereas three of the four mutants sustained promoter activity in serous cells, the M2 mutation did not (Fig. 9), indicating that the M2 mutated sequence is critical for promoter function as well as for protein binding. Promoter activity of the wild type sequence was negligible in fibroblasts; this was unchanged by the M2 mutation.

A nucleotide subsequence search using McVector software revealed that the M2 mutation and surrounding sequence closely resemble the binding site for protein products of the *ets* protooncogene family [McLeod et al., 1992]. The sequence 5′-AGGAAG-3′, a binding site for *ets* family proteins, is present between positions −40/−45 upstream of the cow lysozyme gene and at approximately the same location with respect to the human [Peters et al., 1989a] gene.

DISCUSSION

In tracheobronchial tissue, lysozyme expression is tightly restricted to the serous cell of the submucosal glands and is an integral part of the differentiation program of this cell type [Bowes

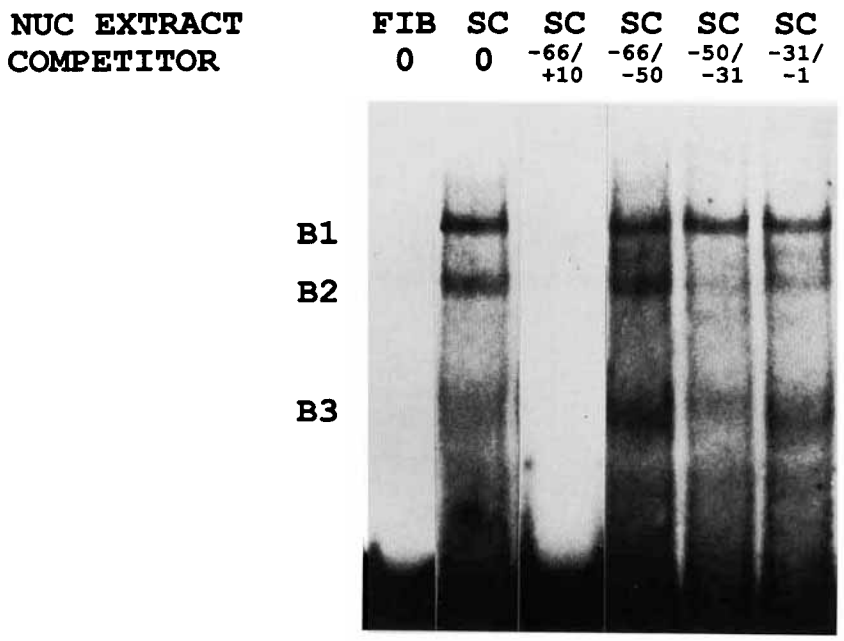

Fig. 7. EMSA analysis of *lys 5a*-binding proteins. Nuclear proteins were incubated with a probe consisting of the ^{32}P-labeled 76-bp fragment (−66 to +10) and subjected to EMSA as described in the text. Probe incubations were with nuclear protein extracts (10 μg) from fibroblasts (FIB) or serous cells (SC). Probe incubations proceeded without (0) or with oligonucleotide competitor corresponding to the sequences indicated.

and Corrin, 1977]. Previously, we showed that this restricted expression is controlled at the level of steady state mRNA [Dohrman et al., 1994], a phenomenon that could involve transcriptional or degradative mechanisms, or both. We report here the results of transient transfection assays indicating the presence, in the 5′ flanking region of a bovine lysozyme gene, of two positive and four negative regulatory elements that could potentially influence serous cell lysozyme expression in vivo. To our knowledge, this is the first information regarding transcriptional control elements of a mammalian, epithelially expressed lysozyme gene (in contrast to extensive information on the chicken lysozyme gene: [Baniahmad et al., 1987; Steiner et al., 1987; Baniahmad et al., 1990; Ahne and Stratling, 1994; and Goethe and Phi van, 1994] and minimal information on the mouse myeloid M-lysozyme gene: [Klages et al., 1992; Möllers et al., 1992].

All constructs tested in our transient transfection assays showed greater transcriptional activity in serous cells than in fibroblasts (Fig. 5). Fibroblasts, which do not express lysozyme in vivo [Hauser et al., 1981] showed weak use of the promoter constructs in vitro. This is consistent with previous reports of weak promoter use by inappropriate cell types in transient transfection assays [Jiang et al., 1993; Zhang et al., 1994a] apparently due to the absence in transfected cells of adjuvant in vivo control mechanisms. Such mechanisms include methylation and chromatin organization as well as the presence of proteins capable of competitively inhibiting the binding of transcription factors to the promoter. The single construct showing the greatest degree of specificity was CATB (−66/+10), which was 10-fold more active in serous cells than in fibroblasts. Further analysis of DNA fragment −66/+10 by EMSA revealed that it bound proteins from serous cell, but not fibroblast nuclear extracts (Fig. 7), consistent with the possibility that serous cell-specific expression is mediated by these proteins.

Initial localization of the protein binding sites was achieved using oligonucleotide subdomains of −66/+10 in binding competition assays. Although formation of protein-DNA complexes B2 and B3 was competitively inhibited by oligonucleotide −50/−31 (Fig. 7), B1 formation was not competitively inhibited by any of the oligonucleotides. This may mean that B1 formation, which was inhibited by competition with the complete fragment −66/+10: (a) occurs through protein-protein interactions requiring a relatively large fragment of DNA; (b) straddles the boundary between oligonucleotides; or (c) re-

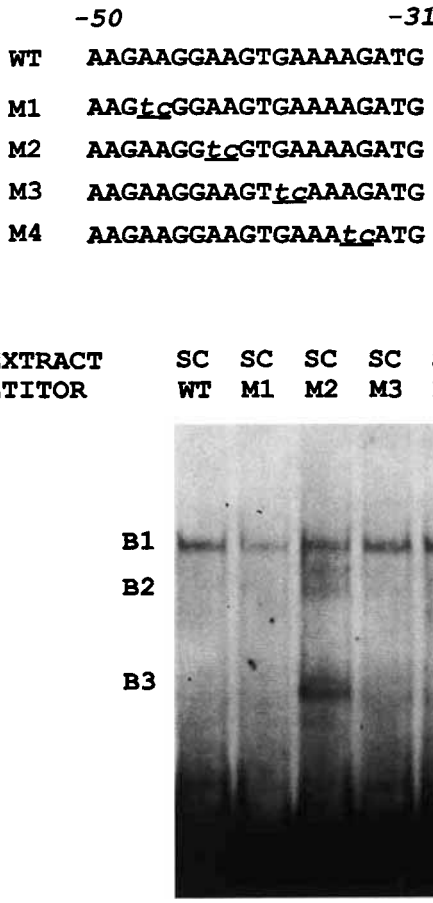

Fig. 8. EMSA analysis of *lys 5a*-binding proteins: localization of binding site for LSF2 and LSF3. Probe incubations were with serous cell nuclear protein extract (10 μg) and oligonucleotide competitors corresponding to the wild type (WT) sequence −50/−31 or mutations M1-M4, as indicated in **upper panel.**

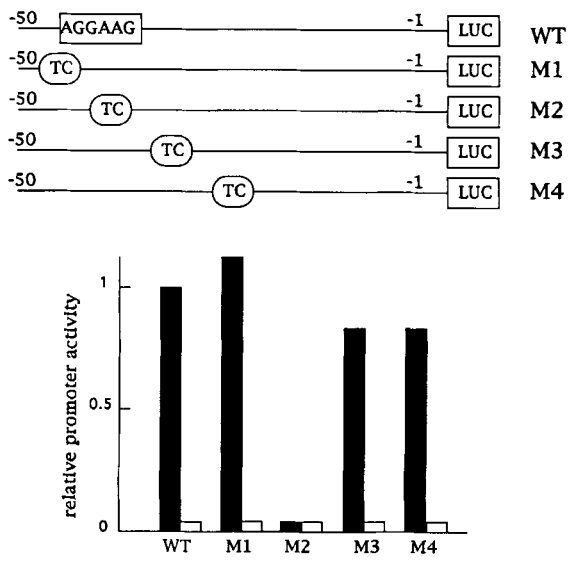

Fig. 9. Analysis of the effect on promoter activity of mutations in the ets protein binding site. **Upper panel:** Fusion constructs of the *lys 5a* 5′-flanking region containing sequential dinucleotide mutations and a luciferase (LUC) reporter gene. **Lower panel:** Transcriptional activity of the mutation-containing constructs are shown relative to a construct containing the wild type (WT) sequence. *Filled bars* = serous cells; *unfilled bars* = fibroblasts. Values are means obtained as for Figure 5.

sides within the coding sequence +1/+10, which was not tested.

More accurate localization of the protein binding sites was achieved by mutation analysis. Results indicated that both B2 and B3 were formed by the binding of proteins to the 6-mer 5′-GGAAGT-3′. This could occur if the protein involved in the B3 complex were a degraded form of that involved in the B2 complex or alternatively if the protein involved in the B3 complex provided a site for the binding of yet another transcription factor which, together with the probe and B3 protein, yielded the lower mobility complex B2.

The GGAAGT motif was not only crucial for protein binding (Fig. 8), but was also crucial for serous cell-specific transcriptional activity (Fig. 9). Sequence comparison revealed similarity between this motif and previously reported binding sites for *ets* family proteins [McLeod et al., 1992; Karim et al., 1990; Nerlov et al., 1991; Brown and McKnight, 1992; Zhang et al., 1994b]. The GGAA purine core known to be essential for specific binding of ets-related proteins [McLeod et al., 1992] is conserved at or near the same location (position −50) in the 5′ flanking region of lysozyme genes of the cow 5a (this report), human [Peters et al., 1989], mouse [Cross et al., 1988], and chicken [Grez et al., 1981] (Fig. 4). A functionally important ets protein binding site was previously seen in an enhancer 2.7 kb upstream of the transcription start site of the chicken lysozyme gene. This enhancer contains a recognition site for the ets family transcription factor PU.1. Mutation of this site abolishes enhancer activity [Ahne and Stratling, 1994].

Despite the apparent presence of ets proteins in fibroblasts [Latinkic and Lau, 1994], we saw no DNA-protein complex formation by EMSA analysis of fibroblast nuclear extracts. This may be due to our use of relatively high ionic strength (complex destabilizing) buffers to facilitate the competition binding assays. These buffers would not favor the survival of complexes formed through low affinity interactions between fibroblast proteins and the promoter.

Amplifying the transcriptional activity of the basal promoter in CATB (−66/+10) was a posi-

tive regulatory element residing between positions −94 and −66. Its ability to modulate the activity of a heterologous (SV40) promoter in a position-independent manner led us to conclude that the region contains an enhancer. The region is well conserved in the human lysozyme gene [Peters et al., 1989a] and contains the sequence TTTCCAC (Fig. 4), previously identified as a binding site for the CAAT enhancer binding protein (C/EBP). The fragment containing this motif increased transcriptional activity of the lysozyme and SV40 promoters in both serous cells and fibroblasts (Figs. 5 and 6). We observed an inverse relationship between promoter strength and potency of modulation by this enhancer, consistent with that demonstrated previously for silencer elements [Baniahmad et al., 1987]. Based on its recognized role in activating genes encoding acute phase reactants such as α1-acid glycoprotein, albumin, and α1-antitrypsin [Friedman and McKnight, 1990; Zhang et al., 1990; Alam and Papaconstantinou, 1992; Chang et al., 1990], C/EBP activation of lysozyme transcription may be part of the acute phase response to infection. In this respect, it is interesting that LPS-mediated activation of lysozyme transcription in macrophages requires an intact C/EBP binding site [Goethe and Phi Van, 1994].

Of the four known isoforms of C/EBP, [Akira et al., 1990; Poli et al., 1990; Descombes et al., 1990; Cao et al., 1991], two are strongly induced by lipopolysaccharide (LPS) [Alam and Papaconstantinou, 1992]. Based on our results showing that a region containing a C/EBP motif can strongly amplify lysozyme promoter activity, it is possible that the observed induction of lysozyme expression by LPS (32; 33) is mediated by C/EBP. Whereas C/EBP has not yet been specifically localized to tracheobronchial serous cells, high levels of both C/EBPb and C/EBPs mRNA have been reported in extracts of lung tissue containing this cell type [Cao et al., 1991].

Opposing the positive regulatory effects of the ets and c/EBP motif-containing elements are four negative regulatory elements, each of which inhibited transcriptional activity in both serous cells and fibroblasts. All of them (−170 to −95, −2500 to −1287, −4600 to −2500, and −6100 to −4600) were silencers based on their orientation- and position-independence and ability to inhibit activity of a heterologous (SV40) promoter (Fig. 6). Silencers have been found near a number of genes [Rosen et al., 1985; Saffer and Thurston, 1989].

The presence of multiple silencers arrayed over several kb in the *lys 5a* gene is reminiscent of the organization of the chicken lysozyme gene, which has been shown to contain 3 major silencers, occurring near positions −0.25, −1.0, and −2.4 kb (for a review see [Sippel and Renakawitz, 1989]). Neither the positions nor the sequences of the silencers are conserved between the chicken and cow.

Silencers might be expected to function more strongly in gene non-expressing than in gene-expressing cells, and in some cases this is true. For example, in the chicken lysozyme gene, the silencers at −2.4 kb [Steiner et al., 1987] and −0.25 kb [Baniahmad et al., 1987] are more active in immature (lysozyme non-expressing) than in mature (lysozyme-expressing) macrophages. In contrast, however, the silencer at −1.0 kb is active in both lysozyme-expressing and non-expressing cells [Baniahmad et al., 1987]. This functional paradox was also described for the silencer termed the rat repetitive element [Laimins et al., 1986], and applies, as well, to all four *lys 5a* silencers, which are essentially equally active in serous cells and fibroblasts (Figs. 5 and 6). It is possible, however, that the observed use of silencers by serous cells is peculiar to the in vitro situation, in which chromatin organization, methylation patterns and proteins capable of competitively inhibiting silencer binding in vivo are absent. Thus, our findings do not exclude the possibility that one or more of the silencers contributes to the maintenance of serous cell-specific expression in vivo.

In summary, our functional analysis of the 5′ flanking region of the bovine lysozyme gene *lys 5a* has yielded evidence for the presence of at least two positive and four negative regulatory elements. The cumulative effect of these yielded greater transcriptional activity in serous cells than in fibroblasts, consistent with in vivo expression patterns. In this regard, it is interesting that the 5′ flanking sequences of other genes showing serous cell-specific expression in vivo resemble that of the lysozyme gene in containing the ets protein binding site AGGAAG crucial to the regulation of *lys 5a* transcription. Specifically, the motif is present in the genes encoding proline-rich protein [Kim and Maeda, 1986], lactoferrin [Johnston et al., 1992], cystic fibrosis transmembrane conductance regulator [Yoshimura et al., 1991], and secretory leukopro-

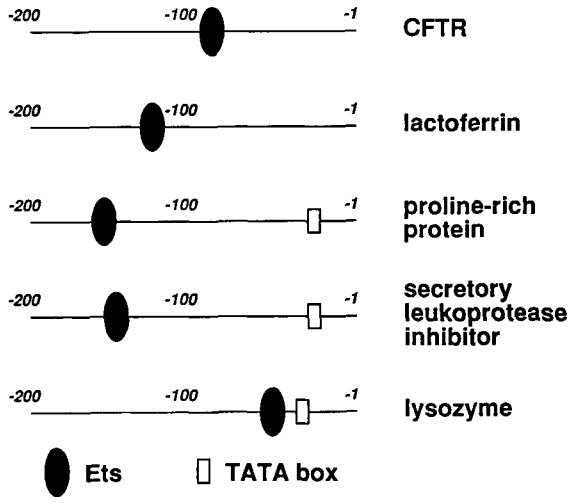

Fig. 10. Diagram indicating the position of ets protein binding sites in the promoters of five genes whose expression in the trachea is restricted to submucosal gland serous cells.

tease inhibitor [Abe et al., 1991] (Fig. 10). 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 [Lassar and Munsterberg, 1994].

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