Cell-type specificity of regulatory elements identified by linker scanning mutagenesis in the promoter of the chicken lysozyme gene

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ABSTRACT

The chicken lysozyme gene is constitutively expressed in macrophages, in oviduct cells its expression is controlled by steroid hormones, and in fibroblasts the gene is not expressed. A fusion gene consisting of promoter sequences of the lysozyme gene from −208 to +15 in front of the chloramphenicol acetyltransferase (CAT) coding region was more than 50 times less active in non-expressing cells as compared to expressing cells. In order to identify the element(s) responsible for this cell-type specificity 31 different linker scanning mutations were generated within this promoter fragment and analyzed by transient transfections in the three types of chicken cells mentioned above. Three mutation sensitive regions located around position −25, −100 and between −158 and −208 were detected in each cell type, however, several LS mutations displayed clear cell-type specific differences in their phenotypic effects. Interestingly, a few LS mutations led to an increase in promoter activity in fibroblasts suggesting that the corresponding wildtype sequences represent binding sites for negatively acting transcription factors.

INTRODUCTION

The chicken lysozyme gene provides an attractive model system to study the mechanisms underlying inducible and tissue-specific expression of eukaryotic genes (see 1 for a review). In tubular gland cells of the chicken oviduct, transcription of the lysozyme gene is induced by steroid hormones (2). In macrophages, the gene is transcribed constitutively at a low level and is not influenced by steroid hormones (3). In fibroblasts, the lysozyme gene is not expressed (own unpublished results).

By gene transfer experiments several distinct regulatory elements have been identified in the 5′-flanking region of the gene. Sequences located at −6.1 kb, −2.7 kb and between positions −208 and −66 specifically enhance transcription of the lysozyme gene in macrophages (4,5), whereas sequences at −2.4 kb, −1.0 kb and between positions −260 and −207 act as silencers (6,4). In addition glucocorticoid and progesterone responsive elements have been identified within the first 208 base pairs upstream of the transcription start site and at −1.9 kb (7,8). Chromatin studies revealed the presence of DNaseI hypersensitive sites at the locations of the various regulatory elements whenever the gene is active thereby confirming the functional significance of the identified control sequences (9). Present knowledge about the chicken lysozyme gene leads to the conclusion that the inducible and cell-type specific expression of the gene is achieved by a complex interplay between several positively and negatively acting regulatory elements dispersed over more than 6kb of 5′-flanking sequences.

Until now very little is known about the internal structure of the chicken lysozyme gene promoter. There is evidence, however, that the promoter contains sequences responsible

for inducibility by glucocorticoids and progestins in oviduct cells and sequences which specifically enhance transcription in macrophages (8,4). Moreover, this region might play an important role in mediating the effects of the various regulatory elements located far upstream of the gene (10).

In order to determine the internal organization of the lysozyme gene promoter, 31 different linker scanning (LS) mutations were generated within a fragment extending from position −208 to +15. LS mutations are ideally suited to analyze a complex eukaryotic promoter containing multiple regulatory elements. Despite its recognized advantages, linker scanning mutagenesis has not been used very frequently because the procedure originally described by McKnight and Kingsbury has proven to be laborious and time consuming (11). We have developed an alternative method which simplifies the construction of LS mutants. A detailed description of this method can be found in a separate publication (12).

Here we present a functional analysis of the lysozyme gene LS mutants in various cell types. Promoter fragments carrying LS mutations were fused to the coding region of the CAT gene. The phenotypic effects of the different LS mutations were monitored in transient transfection experiments using different chicken cells as recipients. The fusion genes were introduced into fibroblasts, macrophages and primary cultures of oviduct cells. We describe the identification of several distinct regulatory elements within the promoter of the chicken lysozyme gene and provide evidence that the promoter is directly involved in the cell-type specific expression of this gene. In addition, we describe an efficient transfection procedure for primary cultures of chicken oviduct cells based upon electroporation of freshly prepared cells. This transfection procedure will be of great use for questions concerning cell-type specificity and hormone inducibility of genes expressed in the chicken oviduct.

MATERIALS AND METHODS

Plasmids

The construction of the different linker scanning mutants has been described in detail elsewhere (12). All other recombinant plasmids were constructed according to standard procedures (13). The lysozyme LS mutants named plysLSn/m were digested with PstI and BamHI. The excised mutated promoter fragments were isolated and ligated into the appropriately cleaved promoterless CAT vector pBLCAT3 (14). The resulting fusion genes were designated plysCATn/m. The variables n and m specify the first and the last nucleotide of the wildtype sequence, that has been substituted by the linker sequence. The plasmid plysCAT-208, which contains the wildtype promoter, was constructed in an analogous way starting from the two plasmids pUClysΔ-208A (12) and pBLCAT3.

The plasmid pRSVCAT/B (R. Mestril, unpublished) consists of the 3.4 kb BglII/BamHI fragment from pRSVgpt (15) and the 1.6 kb BglII/SmaI fragment from pBLCAT3 (14). The BamHI site was filled in. The plasmid pRSVlacZ (W. Ankenbauer, unpublished) consists of the 2.1 kb HindIII/ScaI fragment from pRSVgpt (15) and the HindIII/ScaI fragment from pCH110 (16) containing the lacZ gene. To construct the plasmid pRSVlacZII (W. Ankenbauer, unpublished), the small HindIII/NdeI fragment from pUC8 (17) was exchanged against the RSV LTR containing HindIII/NdeI fragment from pRSVlacZ. In a second step, the HindIII/BamHI fragment from pRSVlacZ carrying the lacZ gene was ligated into the appropriate sites.

All plasmid DNAs used for transfection were purified by two successive CsCl/EtBr gradients.

Cell culture

HD11 cells (=HBC1=LSCC-HD(MC/MA1); 18) and Tasp7 cells (chicken fibroblast cell line transformed by a temperature sensitive mutant of the Rous sarcoma virus, H. Beug, unpublished) were grown in DMEM supplemented with 8% fetal calf serum and 2% chicken serum. The medium contained in addition 10 mM HEPES pH 7.4, penicillin (100 μ/ml) and streptomycin (100mg/ml). HD11 cells were incubated at 37°C, 5% CO_2, Tasp7 cells at 41°C, 5% CO_2. Primary oviduct cells were grown at 41°C, 5% CO_2 in DMEM supplemented with HEPES and antibiotics as above, 8% chicken serum, 2% fetal calf serum and human insulin at a concentration of 5 mg/ml. The sera were treated with charcoal before use to remove endogenous steroid hormones.

Preparation of primary oviduct cells

Two or three 4 cm long silicon tubes (inner diameter 1.47 mm; wall thickness 0.245 mm; Dow Corning) each filled with about 40 mg diethylstilbestrol and closed with adhesive silicone (Dow Corning) were subcutaneously implanted in the left groin region of 3 week old female chickens (strain 'Brown Leghorn') (19). Two to three weeks later the tubes were removed. Two days after the removal, the animals were decapitated and the oviducts were taken aseptically. The tissue was finely minced with scissors in phosphate buffered saline (PBS), washed several times with the same buffer and finally suspended in DMEM containing 10 mM HEPES as well as collagenase, hyaluronidase and dispase each at a concentration of 0.5 mg/ml. The enzymatic disintegration of 2–3 oviducts was performed in a 100 ml glass bottle in a total volume of 40 ml by incubating the vessel for 1 to 2 hours in a shaking water bath at 37°C. The resulting cell clumps consisting of about 10–100 cells were pelleted, washed with PBS and purified by Percoll (Pharmacia) gradient centrifugation. The tubular gland cells, which banded at about 60% Percoll, were collected, washed several times with PBS and used immediately for electroporation.

DNA Transfection

Primary oviduct cells, HD11 and Tasp7 cells were transiently transfected by electroporation (20) using a 'Gene Pulser' apparatus with a capacitance extender (Bio-Rad). Twenty microgram test plasmid DNA and, if the experiment was internally controlled, 4mg pRSVlacZII, were dissolved in 100 ml TE buffer and added to $1-2\times10^7$ cells in 400 ml PBS. The mixture was pipetted into a 'Gene Pulser' cuvette (Bio-Rad, Cat. No. 165-2065) and immediately electroporated (960 mF, 250 V). Ten minutes after the shock, the cells were removed from the cuvette and transferred to a 10 cm dish containing the appropriate medium.

CAT assay

CAT assays were performed as described (21) with the following modifications: The cells were harvested 16–18 hours after electroporation and cell extracts (100ml) were prepared by 3 cycles of freezing and thawing. The CAT activities of different extracts were determined using equivalent amounts of protein or β-galactosidase activity per sample. The assays were quantitated by liquid scintillation counting.

β-galactosidase assay

β-galactosidase assays were performed as follows: 1 ml Z-buffer (100 mM Na-phosphate pH 7, 10 mM KCl, 1mM $MgSO_4$, 50 mM β-mercaptoethanol) was added to 10 ml cell extract and preincubated for 5 min at 37°C. The reaction was started by adding 200 ml o-nitrophenyl-β-D-galactopyranoside (4 mg/ml in 100 mM Na-phosphate pH 7) and incubated at 37°C until the sample turned yellow. Each sample of a series was incubated

for the same time interval. The reaction was stopped by adding 900 ml 1 M Na_2CO_3 and quantitated by measuring the absorbance at 420 nm.

RESULTS

The chicken lysozyme gene promoter controls CAT gene expression in a cell-type specific manner.

Previous experiments have suggested, that the promoter of the chicken lysozyme gene might be directly involved in the cell-type specific expression of the gene (4). To clarify this point, we have constructed a fusion gene containing lysozyme gene promoter sequences from −208 to +15 in front of the coding region of a CAT reporter gene. A restriction map of this plasmid designated plysCAT-208 is shown in Fig. 1. The plasmid plysCAT-208 and the control plasmids pSV2CAT (21, CAT gene under the control of the SV40 early promoter/enhancer), pRSVCAT/B (CAT gene under the control of the long terminal repeat of the Rous sarcoma virus) and pBLCAT3 (14, CAT gene without promoter) were transiently transfected into three different types of chicken cells. We used Tasp7 cells, a fibroblast cell line which does not express the endogenous lysozyme gene, HD11 cells, a macrophage cell line which constitutively expresses the endogenous gene, and primary cultures of oviduct cells in which the gene can be induced by steroid hormones.

Gene transfer experiments employing chicken oviduct cells have been problematic. Since no cell line is available, primary cultures have to be used, which cannot be efficiently transfected by the calcium phosphate or the DEAE dextran procedure (22). Until now microinjection has mainly been used to introduce DNA into these cells (22,23). Since this method is not appropriate to study the effects of mutations quantitatively, an alternative

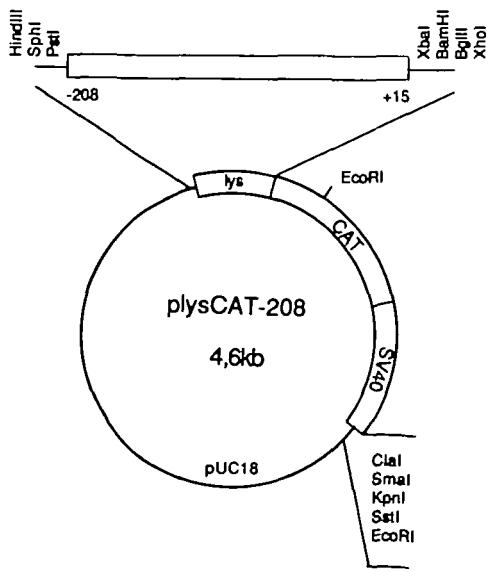


Figure 1: *Restriction map of the plasmid plysCAT-208.* Promoter sequences of the chicken lysozyme gene between −208 and +15 are marked by 'lys', the coding region of the chloramphenicol acetyltransferase gene by 'CAT', and the splicing and polyadenylation signals from the SV40 virus by 'SV40'.

transfection procedure had to be established. Electroporation, which has successfully been applied to cells resistant to transfection by the standard calcium phosphate or DEAE dextran method (24), proved to be well suited for our purpose. However, even with electroporation the absolute CAT values for plysCAT-208 and the two external standards pSV2CAT and pRSVCAT/B were much lower in primary oviduct cells than in HD11 macrophages reflecting most likely the different transfection efficiencies of these cells. Internally controlled transfection experiments with plysCAT-208, pBLCAT3 and pSV2CAT do also support this view (data not shown).

The results of the transfection experiments are depicted in Fig. 2. As expected, the control plasmids pSV2CAT and pRSVCAT/B were strongly expressed in all 3 cell types. The plasmid plysCAT-208 exhibited a similar level of CAT activity as either pSV2CAT or pRSVCAT/B in oviduct cells and macrophages. In fibroblasts, however, the expression of plysCAT-208 was more than 50-fold lower than that of the two control plasmids. The promoterless plasmid pBLCAT3 displayed in all 3 cell types only residual CAT activity indicating that CAT activity is based on transcripts initiated at the correct start site of transcription within the respective promoter. This has previously been confirmed by RNA start site mapping experiments employing lysozyme-CAT fusion genes almost identical to ours (6). From these data we conclude that the activity of the lysozyme gene promoter is regulated in a cell-type specific manner.

A set of linker scanning mutants spanning the promoter of the chicken lysozyme gene. In order to identify the element(s) responsible for the observed cell-type specificity, we have generated by a novel method 31 different linker scanning mutations within the corresponding promoter fragment of the lysozyme gene extending from position −208 to +15. A detailed description of this mutagenesis procedure has been published elsewhere (12). The sequences of the different LS mutations are shown in Fig. 3.

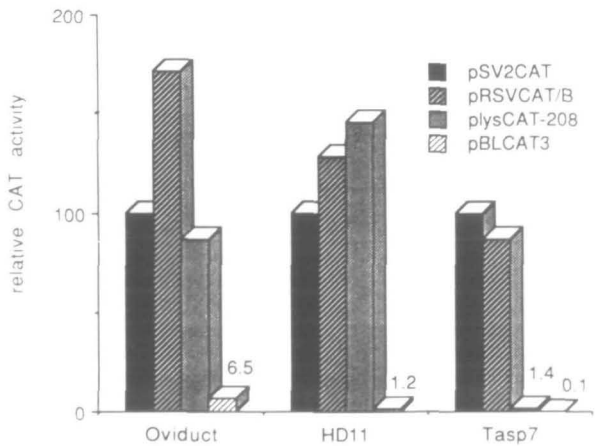
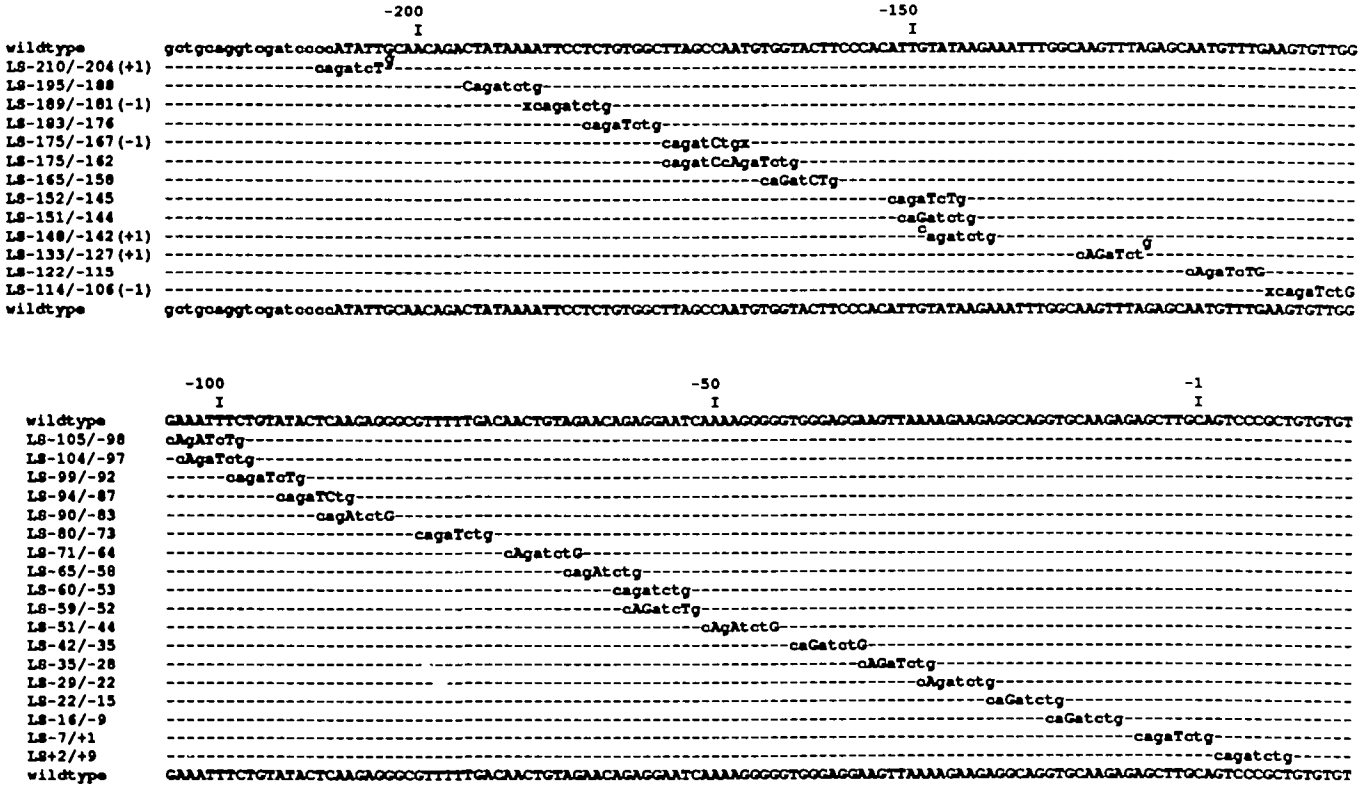


Figure 2: Cell-type specificity of the promoter of the chicken lysozyme gene. Primary cultures of chicken oviduct cells, HD11 macrophages, and Tasp7 fibroblasts were transfected with the plasmids pSV2CAT, pRSVCAT/B, plysCAT-208 and pBLCAT3. Cell extracts were prepared and assayed for CAT enzyme activity. The values are expressed relative to pSV2CAT, which has been given the arbitrary value of 100. The absolute CAT values for plysCAT-208 are given in the legends to figures 4−6. The diagram shows the mean values of 3 independent experiments.


-200
-150
wildtype gctgcaggtcgatccccATATTGCAACAGACTATAAAATTCCTCTGTGGCTTAGCCAATGTGGTACTTCCCACATTGTATAAGAAATTTGGCAAGTTTAGAGCAATGTTTGAAGTGTTGG
LS-210/-204(+1) ----------cagatcTg----------
LS-195/-188 ----------Cagatctg----------
LS-189/-181(-1) ----------xcagatctg----------
LS-183/-176 ----------cagaTctg----------
LS-175/-167(-1) ----------cagatCtgx----------
LS-175/-162 ----------cagatCcAgaTctg----------
LS-165/-158 ----------caGatCTg----------
LS-152/-145 ----------cagaTcTg----------
LS-151/-144 ----------caGatctg----------
LS-148/-142(+1) ----------cagatctg----------
LS-133/-127(+1) ----------cAGaTct----------
LS-122/-115 ----------cAgaTcTG----------
LS-114/-106(-1) ----------xcagaTctG
wildtype gctgcaggtcgatccccATATTGCAACAGACTATAAAATTCCTCTGTGGCTTAGCCAATGTGGTACTTCCCACATTGTATAAGAAATTTGGCAAGTTTAGAGCAATGTTTGAAGTGTTGG
-100
-50
-1
wildtype GAAATTTCTGTATACTCAAGAGGGCGTTTTTGACAACTGTAGAACAGAGGAATCAAAAGGGGGTGGGAGGAAGTTAAAAGAAGAGGCAGGTGCAAGAGAGCTTGCAGTCCCGCTGTGTGT
LS-105/-98 cAgATcTg----------
LS-104/-97 -cAgaTctg----------
LS-99/-92 ------cagaTcTg----------
LS-94/-87 ----------cagaTCtg----------
LS-90/-83 ----------cagAtctG----------
LS-80/-73 ----------cagaTctg----------
LS-71/-64 ----------cAgatctG----------
LS-65/-58 ----------cagAtctg----------
LS-60/-53 ----------cagatctg----------
LS-59/-52 ----------cAGatcTg----------
LS-51/-44 ----------cAgAtctG----------
LS-42/-35 ----------caGatctG----------
LS-35/-28 ----------cAGaTctg----------
LS-29/-22 ----------cAgatctg----------
LS-22/-15 ----------caGatctg----------
LS-16/-9 ----------caGatctg----------
LS-7/+1 ----------cagaTctg----------
LS+2/+9 ----------cagatctg------
wildtype GAAATTTCTGTATACTCAAGAGGGCGTTTTTGACAACTGTAGAACAGAGGAATCAAAAGGGGGTGGGAGGAAGTTAAAAGAAGAGGCAGGTGCAAGAGAGCTTGCAGTCCCGCTGTGTGT

The promoter fragments carrying different LS mutations were subsequently fused to the coding region of the bacterial CAT gene. This widely used approach has allowed us to measure the effects of the individual LS mutations in a simple, rapid and sensitive way (21). To facilitate the generation of the lysozyme-CAT fusion genes, we have constructed the promoterless CAT vector pBLCAT3 which contains a polylinker with 8 unique restriction sites upstream of the CAT gene (14). The new plasmids were designated plysCATn/m, whereby n and m denote the position of the first and the last nucleotide in the lysozyme wildtype sequence that has been exchanged with the linker sequence. Apart from the LS mutation in the promoter region, all plasmids of the plysCATn/m series and the wildtype plasmid plysCAT-208 are identical.

Functional analysis of linker scanning mutations in the promoter of the chicken lysozyme gene in various types of chicken cells.

The phenotypic effects of the different LS mutations were analyzed by transiently transfecting the plasmids of the plysCATn/m series into the same cell types (primary oviduct cells, HD11 macrophages and Tasp7 fibroblasts) in which the wildtype plasmid plysCAT-208 had been tested before. In a typical experiment one sample of each of the 31 mutants, three samples of the wildtype plysCAT-208, one sample of pBLCAT3 and one sample without DNA were transfected. Approximately 18 hours after transfection cell extracts were prepared and assayed for CAT activity.

Fig. 4, 5 and 6 show the results of several transient transfection experiments using primary oviduct cells, HD11 macrophages and Tasp7 fibroblasts as recipients. These data demonstrate that the promoter of the chicken lysozyme gene contains at least 3 mutation sensitive regions. These regions are located around position −25, −100 and between −158 and −208. Most mutations influencing the activity of the promoter led to a reduction, whereas some mutations caused an increase in promoter activity. Many mutations within the sensitive areas caused similar effects in all 3 cell types, however, some mutations clearly showed cell-type specific differences. For example, the mutant LS-210/-204 showed wildtype activity in oviduct cells, 4-fold reduced activity in fibroblasts and more than 30-fold reduced activity in macrophages. Several other mutants (LS-16/-9, LS-22/-15 or LS-183/-176) also showed cell-type specific differences. LS-16/-9 and LS-22/-15 exhibited nearly wildtype activity in oviduct cells, a 1.5-fold increased activity in macrophages and a 4-fold increased promoter activity in fibroblasts. LS-183/-176 showed wildtype activity in oviduct cells, but a 2.5-fold higher activity in macrophages as well as fibroblasts.

It is interesting that in primary oviduct cells none of the 31 different LS mutations caused an increase in promoter strength, whereas in macrophages one (LS-183/-176) and in fibroblasts at least three mutations (LS-16/-9, LS-22/-15, LS-183/-176) increased the activity of the promoter.

DISCUSSION

The experiments described in this report clearly show that a promoter fragment of the chicken lysozyme gene extending from position −208 to +15 contains regulatory sequences involved in the cell-type specific expression of this gene. The promoter was highly active in macrophages and primary oviduct cells and showed only residual activity in fibroblasts.

Figure 3: Nucleotide sequences of LS mutations in the promoter of the chicken lysozyme gene. The sequences of 31 different LS mutations and the wildtype sequence between position −208 and +15 are shown. The sequence of the BglII linker is indicated; mutated nucleotides are indicated with small letters, wildtype nucleotides with capital letters, deleted nucleotides are indicated by an x, and inserted nucleotides are placed above the stippled line.

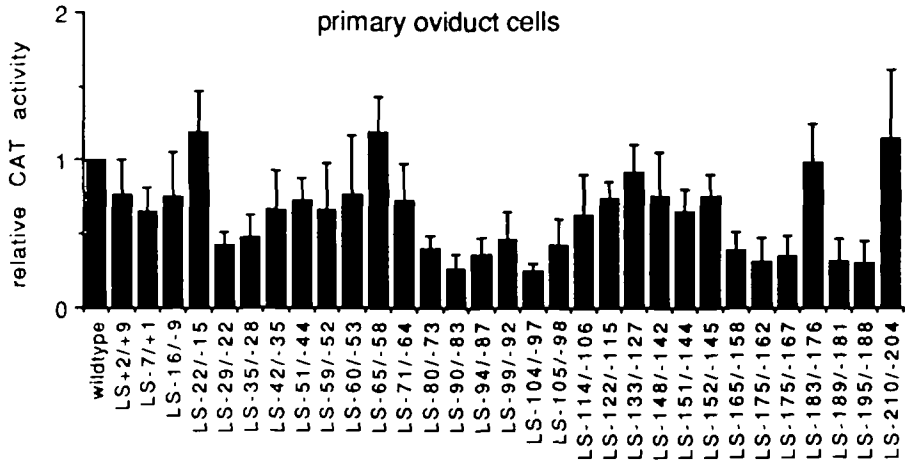


Figure 4: Activity of different LS mutants in primary chicken oviduct cells. Primary oviduct cells were transfected with the indicated plysCATn/m plasmids or plysCAT-208 (wildtype) and assayed for CAT enzyme activity. The values are expressed relative to the wildtype (approx. 100 pmol min^{-1} mg^{-1}), which has been given the arbitrary value of 1. The diagram shows for each LS mutant the mean value and the standard deviation of its relative activity calculated from 6 independent experiments. In 3 experiments pRSVlacZII was cotransfected as an internal control and used to normalize the CAT assays.

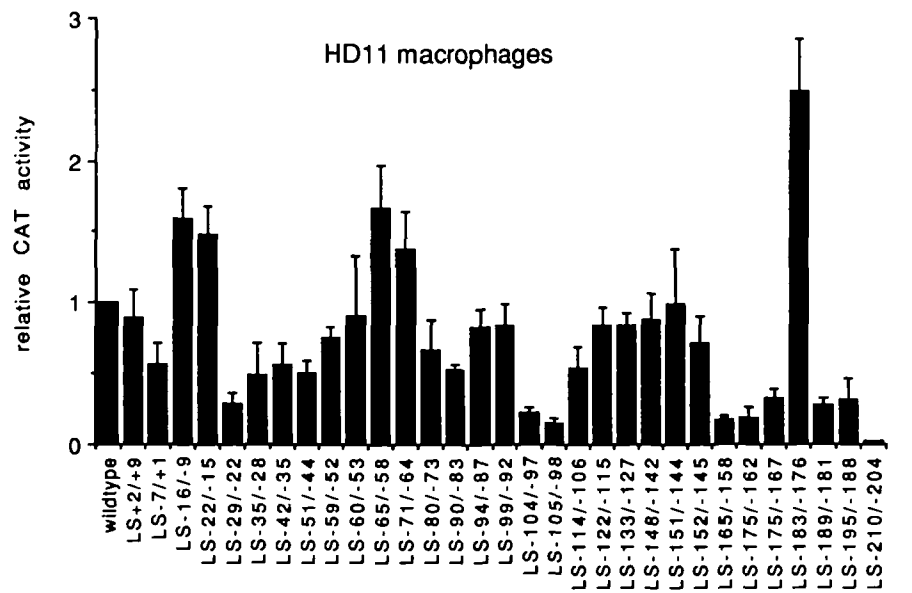


Figure 5: Activity of different LS mutants in the chicken macrophage cell line HD11. HD11 cells were transfected with the indicated plysCATn/m plasmids or plysCAT-208 (wildtype) and assayed for CAT enzyme activity. The values are expressed relative to the wildtype (approx. 3000 pmol $min^{-1} \cdot mg^{-1}$), which has been given the arbitrary value of 1. The diagram shows for each LS mutant the mean value and the standard deviation of its relative activity calculated from 3 independent experiments. In two experiments pRSVlacZII was cotransfected as an internal control and used to normalize the CAT assays.

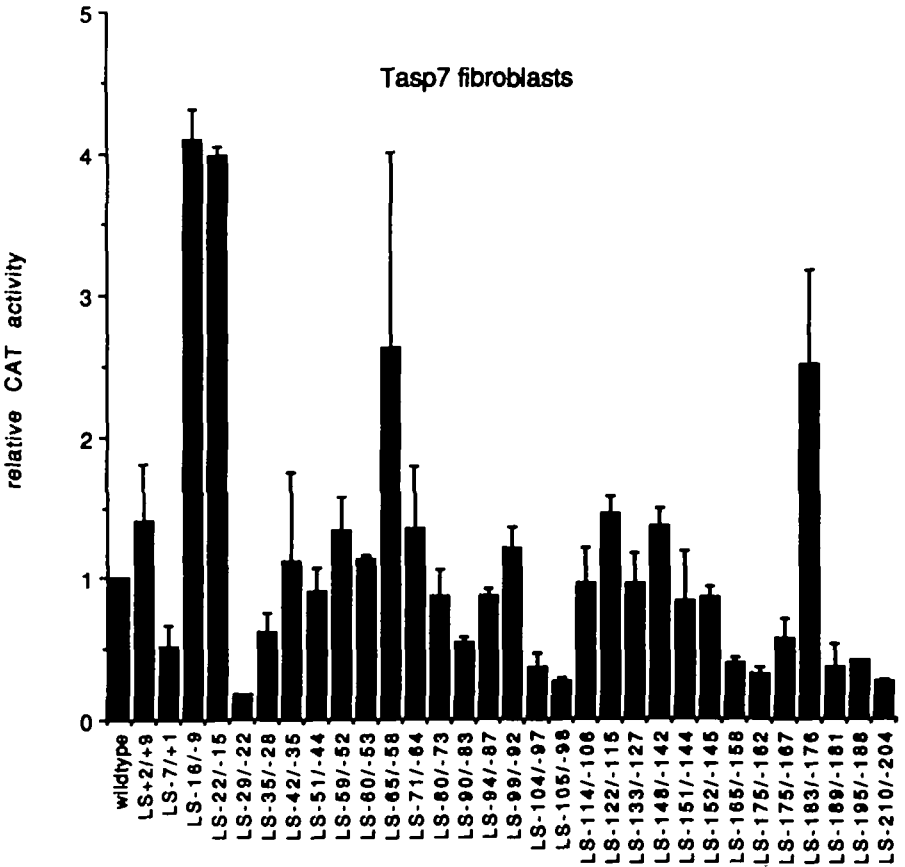


Figure 6: Activity of different LS mutants in the chicken fibroblast cell line Tasp7. Tasp7 cells were transfected with the indicated plysCATn/m plasmids or plysCAT-208 (wildtype) and assayed for CAT enzyme activity. The values are expressed relative to the wildtype (approx. 50 pmol·min^{-1}·mg^{-1}), which has been given the arbitrary value of 1. The diagram shows for each LS mutant the mean value and the standard deviation of its relative activity calculated from two independent experiments. In both experiments pRSVlacZII was cotransfected as an internal control and used to normalize the CAT assays.

These data obtained from transient transfection experiments correlate well with the expression pattern of the endogenous lysozyme gene. The promoters of the chicken ovalbumin and conalbumin gene, two other egg white protein genes, are also only active in chicken oviduct cells but not in chicken fibroblasts or kidney cells and a variety of non-chicken cells (22). Microinjection experiments using lysozyme-SV40-T-antigen fusion genes (23) and transient transfection experiments employing lysozyme-CAT fusion genes (4) also support the idea of a cell-type specific promoter. It should be stressed, however, that the promoter is not the only region involved in cell-type specificity of the lysozyme gene. Sequences located as far upstream of the gene as −2.7 kb or −6.1 kb also play a role in this respect (4,5).

Surprisingly, in macrophages and oviduct cells the lysozyme gene promoter fragment

present in plysCAT-208 is as strong as the SV40 early promoter/enhancer or the RSV promoter/enhancer, which both have been shown to be highly active in a wide variety of eukaryotic cells (25,26). This high activity could be due to the fact that the 3 silencers identified within the 5'-flanking sequences of the chicken lysozyme gene (6,4) are not present in plysCAT-208. A similar type of regulation has been described for the chicken ovalbumin gene. The promoter of this gene is normally repressed, but deletion of a negative element located between −425 and −132 leads to a high transcriptional activity in oviduct cells (27).

The functional analysis of the LS mutants demonstrates that the promoter of the chicken lysozyme gene is composed of several sequence motifs whose integrity is required for full promoter activity. The phenotypic effects of individual LS mutations were usually moderate. With one exception, no single LS mutation completely abolished promoter activity. This observation is in full agreement with similar studies on other promoters (11,28,29). The analysis of the lysozyme gene promoter revealed at least 3 different mutation sensitive regions. Most mutations in the sensitive areas led to a reduction of the promoter activity, but interestingly, some mutations also increased the promoter strength. This increase could be explained either by the destruction of a repressor binding site, by the generation of a new or improved binding site for a positively acting transcription factor or by the optimation of a facet of promoter geometry, such as DNA curvature.

The first mutation sensitive region was located about 25 bp upstream of the transcription start site. It spanned the sequence −32 TTAAAAGAAGA −22, which is not very typical for a TATA box. Nevertheless, the position and the A/T content of this sequence imply that it represents the TATA box of the lysozyme gene promoter. The two mutations LS-22/-15 and LS-16/-9, located adjacent to the TATA box, displayed a remarkable cell-type specific pattern of activity. They exhibited wildtype activity in primary oviduct cells, slightly increased activity in macrophages and 4-fold increased activity in fibroblasts. They were the strongest 'up' mutants of the entire series. At the present time it is unclear, which of the possibilities discussed above is responsible for the increase in CAT activity observed in fibroblasts.

The second mutation sensitive region was located about 100 bp upstream of the transcription start site. Mutations in this area led in all 3 cell types to a 4- to 6-fold reduction of CAT activity. Interestingly, the lysozyme gene sequence (−108 TGGGAAATTTCT −97) shows a clear homology to the so called 'positive regulatory domain II' within the promoter of the human β-interferon gene (−65 TGGGAAATTCCT −54, 30). Both sequences are weakly homologous to the 'enhancer core sequence' (31).

The third mutation sensitive region included the sequences between −158 and −208. The size of this region and the results of the transfection experiments allow the conclusion that it is composed of several different regulatory elements. The sequence centered around position −170 shows homology to the CCAAT box (32). The transfection data demonstrate, that this sequence is important for full activity of the lysozyme gene promoter.

The sequence between −190 and −180 (AAATTCCTCTG) shows a perfect homology to a portion (−61 to −51) of the so called 'negative regulatory domain' of the human β-interferon gene (30). Mutation of this homology in the lysozyme gene has no effect in oviduct cells, but increases CAT activity in macrophages and fibroblasts 2.5-fold. This observation offers a possible explanation why the lysozyme gene cannot be induced by glucocorticoids in macrophages although these cells do contain the necessary receptors (33,34). The binding site for the glucocorticoid receptor within the lysozyme gene promoter

(35) would overlap with the potential negative element around −185 and could therefore be blocked by a repressor. Further experiments are required to verify this hypothesis. Such mutually exclusive interactions between different proteins binding to overlapping sequences have not only been described for the human β-interferon gene (36,30), but also for a sea urchin histone gene (37) and the human chorionic gonadotropin gene (38,39).

Around position −190 the sequence 5′-TATAAAA-3′ resembling a TATA box can be found. Mutation of this sequence reduces promoter activity. It is known, that the chicken lysozyme gene uses multiple start sites of transcription, however no transcripts originating further upstream than −58 have been found (40). Therefore, the sequence around position −190 probably does not represent a functional TATA box.

The sequence centered around position −205 does not show any convincing homology to known regulatory sequences. The LS mutation changing this particular sequence (LS-210/-204) showed a very interesting phenotype. It had no effect in oviduct cells, caused a moderate decrease of promoter activity in fibroblasts and led to a dramatic decrease in macrophages. LS-210/-204 was the only mutant of the entire series displaying a clearly different phenotype in each cell type. The transfection data can be explained by assuming that no protein binds to this sequence in oviduct cells, whereas in macrophages and fibroblasts different proteins might bind.

The analysis of the internal organization of the chicken lysozyme gene promoter by linker scanning mutagenesis has revealed a complex modular structure. Clear cell-type specific differences between two expressing cell types on one hand and between the expressing and the non-expressing cell types on the other hand have been observed. The data suggest, that repressors might play an important role in modulating the activity of the lysozyme gene promoter in macrophages and fibroblasts.

During the preparation of this manuscript a paper has been published by Altschmied et al. (41) which presents data nicely complementing our own results. Using nuclear extracts from HD11 cells Altschmied and colleagues have identified by DNaseI footprinting experiments six binding sites for proteins on the lysozyme gene promoter. Five of these footprints exactly coincide with mutation sensitive regions which we have identified by linker scanning mutagenesis.

In the present study, we have concentrated on the identification of regulatory elements residing in the promoter and being responsible for its basal activity in different cell types. Further experiments are required to characterize the proteins involved. Additional investigations will also address the interactions between the promoter and the various elements located further upstream with respect to cell-type specificity and hormonal control. The set of LS mutants will be very useful for these future analyses.

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