
Lysozyme gene activity in chicken macrophages is controlled by positive and negative regulatory elements

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ABSTRACT

The chicken lysozyme gene is constitutively active in macrophages and under the control of steroid hormones in the oviduct. To investigate which DNA elements are involved in the control of its expression in macrophages we performed transient DNA transfer experiments with two different types of plasmids: 5'-deletion mutants of the upstream region of the chicken lysozyme gene and different fragments from this area in front of the thymidine kinase promoter (herpes simplex virus), each placed in front of the CAT (chloramphenicol acetyl transferase) coding sequence. Two enhancers (E-2.7kb and E-0.2kb) were characterized. They are active in macrophages, but not in chicken fibroblasts. Furthermore a negative element (N-2.4kb) was identified, which is active in fibroblasts and promyelocytes, but not in mature macrophages. The combined action of all three elements contributes to the observed lysozyme gene activities: no activity in fibroblasts, moderate activity in promyelocytes and high activity in mature macrophages.

INTRODUCTION

Lysozyme, a specific differentiation marker for the myeloid lineage of hematopoietic differentiation in mammals (1), is also expressed in chicken macrophages (2). The inactive chicken lysozyme gene is activated during macrophage differentiation. Constitutive expression increases from myeloblasts to mature macrophages (2). In addition to this constitutive expression the chicken lysozyme gene is inducible by steroid hormones in the tubular gland cells of the oviduct (3). This steroid induction is mediated by DNA sequences in the immediate vicinity of the promoter (4,5). Lysozyme mRNA in both chicken macrophages and oviduct cells derives from the same lysozyme gene and initiates at identical start sites (2). Here we wanted to identify the control regions responsible for the macrophage specific expression.

Using the DNA transfer technique tissue specific enhancer elements have been identified for several genes, including those for immunoglobulin (6,7,8), chymotrypsin, amylase and insulin (9), albumin (10), muscle actin (11), α -fetoprotein (12,13), fibronin (14) and elastase (15). In the case of the chicken lysozyme gene a macrophage specific enhancer sequence at -6.1kb 5' of the transcriptional start site has been identified (16). Since this

enhancer is active in a myeloid cell line, which synthesizes about one third of the lysozyme amount found in mature macrophages (16), we wanted to know whether additional regulatory elements contribute to the lysozyme gene activity found in mature macrophages. Here we describe two enhancer elements and one negative element, all of which show a tissue specificity. Activity of all three elements in the myeloid cell line leads to a moderate activity of a transfected reporter gene; in mature macrophages a high expression is observed, since the negative element is inactive while the enhancer sequences are active.

MATERIALS AND METHODS

Plasmids

Plasmids were constructed by standard techniques (17). Sense or antisense orientations are indicated by "s" or "a", respectively. Deletion end points and restriction fusion sites were verified by sequencing (18) except for deletion end points further upstream than -380 bp. The DNA sequence of the enhancer fragment -2.71kb/-2.54kb and the negatively acting fragment -2.54kb/-2.25kb was determined for both strands. Before transfection all DNA samples were purified by two centrifugations in CsCl gradients and analyzed on agarose gels to assess the purity and quality of supercoiled DNA. All lysozyme fragments were isolated from subclones of λ lys30 (19).

5' Deletion Series (plysCATA-n). 5' deletion mutants (4) were cloned with their HindIII sites and their filled in EcoRI site into the HindIII and filled in BglII sites of vector ptkCAT3 (20), thereby removing the tk (thymidine kinase) fragment.

plys-4.60/-2.71s tkCAT; plys-4.60/-2.71a tkCAT; plys-2.71/-1.42s tkCAT; plys-2.71/-1.42a tkCAT. The two HindIII lysozyme gene fragments (-4.60kb/-2.71kb and -2.71kb/-1.42kb) were ligated into the unique HindIII site of pBL-CAT2 at position -109 of the tk-promoter. pBL-CAT2 is a vector containing the CAT gene (chloramphenicol acetyl transferase) driven by the tk promoter (B.Luckow and G.Schütz, unpublished). This vector contains convenient polylinker sequences 5' and 3' of the tk-CAT fusion gene.

plys-2.71/-2.25s tkCAT. pBL-CAT2 was linearized with XbaI, filled in, recut with HindIII and ligated with the 465bp long HindIII/Scal lysozyme fragment (-2.71kb/-2.25kb).

plys-2.54/-2.25s tkCAT; plys-2.54/-2.25a tkCAT. The 300bp lysozyme gene fragment BglII/Scal (-2.54kb/-2.25kb) was cloned into the BglII/SmaI sites of pIC19H (21). For integration in both orientations in front of the tk-promoter, the fragment was removed from the pIC vector either by HindIII and BamHI or by HindIII and BglII digestion and ligated into the HindIII plus BamHI opened vector pBL-CAT2.

plys-2.54/-2.34s tkCAT. The 205bp BglII/PvuII lysozyme fragment was cloned into the BglII/SmaI sites of pIC19H, removed again by digestion with BamHI and HindIII and inserted into the HindIII plus BamHI opened vector pBL-CAT2.

plys-2.71/-2.54s tkCAT. The 165bp HindIII/BglII lysozyme fragment was ligated into the HindIII and BamHI digested vector pBL-CAT2 in sense orientation.

plys-2.71/-2.54a tkCAT. After digestion of pBL-CAT2 with BamHI plus BglII the tk-promoter was replaced by the BglII fragment from plys-2.71/-1.42a tkCAT containing lysozyme gene upstream sequences -2.54kb/-2.71kb in addition to the tk-promoter (-109bp to +51bp).

plys-2.69/-2.54s tkCAT; plys-2.63/-2.54s tkCAT. Lysozyme sequences -2.69/-2.54 (148bp RsaI/BglII fragment) and -2.63/-2.54 (87bp SspI/BglII fragment) were cloned into the HindIII and filled in XbaI sites of vector pBL-CAT2.

ptkCATlys-2.71/-2.54a. Integration of the 165bp HindIII/BglII (-2.71kb/-2.54kb) lysozyme fragment behind the tkCAT sequences within the vector pBL-CAT2 was achieved by subcloning this fragment into pIC20R (21), subsequent isolation as a SstI fragment and integration into the SmaI site of pBL-CAT2.

plys-2.71/-2.63s tkCAT. The 78bp HindIII/SspI lysozyme fragment was ligated into the XbaI (filled in) and HindIII sites of vector pBL-CAT2.

plys-1.42/-254s tkCAT. The -1.42kb/-254bp lysozyme fragment was isolated by partial RsaI digestion followed by HindIII digestion and cloned into the HindIII and HindII sites of the vector pUC19 (22). This fragment was again removed by digestion with HindIII and BamHI and inserted into the vector pBL-CAT2 using the identical cloning sites.

plys-208/-66a tkCAT. This plasmid, a gift of R. Miksicek and G. Schütz, contains lysozyme sequences from -208bp to -66bp in antisense orientation in front of position -109bp of the tk promoter.

pE-2.7kbΔ-208CAT. The 165bp HindIII/BglII fragment was isolated from plys-2.71/-2.25s tkCAT and integrated into the plasmid plysCATΔ-208 by replacing the polylinker from the HindIII to the BamHI restriction sites.

pSVtkCAT(Constructed by J.Altschmied). The 72 bp repeats of the SV40 enhancer were excised as a BamHI fragment from ptkCAT14C (gift of R.Miksicek and G.Schütz) and inserted into the unique BamHI site of pBL-CAT2.

pGEM1tkCAT. This *in vitro* transcription vector was generated by inserting an EcoRI fragment of pBL-CAT2 (fragment -80bp to +323bp of the tk-CAT fusion gene) in antisense orientation into the EcoRI site of pGEM1 (Promega Biotec).

Cell Culture

HD11 (= HBC1 = LSCC-HD(MC/MA1)) cells (23) and CEF38 cells (24) were grown in standard growth medium (Dulbecco's modified Eagle's medium (DMEM, Biochrom) supplemented with 8% fetal calf serum (Biochrom), 2% chicken serum (Gibco), and 10 mM Hepes, pH 7.4. The medium contained 100 units/ml penicillin and 100 µg/ml streptomycin.

For continuous growth, the cells were diluted between three and ten fold every three days. Bone marrow primary macrophages were prepared from 7-12 days old White Leghorn chicks as described by Graf (25). The bone marrow cells were seeded on 14.5 cm dishes (Falcon) in growth medium (see above). After one day the fibroblast-depleted supernatant was distributed on fresh dishes and incubated for 6 days. The supernatant was removed and the adherent macrophages were kept in culture for further 7 days, trypsinized and seeded out on 6 cm dishes for DNA transfections (2 d before transfer).

DNA Transfection

All transfections were carried out in triplicate experiments and repeated at least twice. One day prior to transfection, cells of cell lines were seeded on 6 cm dishes (Falcon) at 1×10^6 cells/plate (HD11) or 1×10^5 cells/plate (CEF38). Medium was changed 4 hrs before adding the DNA to the cells. For transient expression 1 pmol of superhelical DNA was transfected per dish by the calcium phosphate method (26). After 20 hrs at 37°C the precipitates were removed, the cells washed, and in the case of HD11 cells additionally shocked for 3 min with 1 ml 15% glycerol in 1xHBS. Cells were fed with fresh medium 24 hrs before harvesting. Calcium phosphate transfections of primary macrophages were performed ten days after isolation on 6 cm dishes according to Wigler et al. (27). For transfection 1.5 pmol plasmid DNA was filled up with high molecular weight calf thymus DNA to a total amount of 15 µg per sample. After 20 hrs of exposure to DNA, cells were shocked for 3 min with 1 ml 15% glycerol in 1x HBS and fed with fresh medium 24 hrs before harvesting.

CAT Assay

CAT assays were performed according to Gorman et al. (28) with modifications: Cells were disrupted by 3 cycles of freezing and thawing. After centrifugation the protein concentration of the supernatant was measured and identical protein amounts per sample were used for the enzymatic reaction.

Preparation of Total RNA

Total cellular RNA was extracted by the guanidinium isothiocyanate procedure as described (29,17). The RNA was prepared from 10^7 cells 48 hrs after transfection with a chimeric CAT construct. Cells transfected in parallel were harvested at the same time and used for CAT assays as described above.

Preparation of Complementary RNA Probes

The template plasmid pGem1tkCAT was linearized with BamHI, phenol extracted and ethanol precipitated. Transcription was carried out for 1 hour at 40°C under the conditions described by Melton et al. (30), using 1 unit/µl of T7 phage RNA-polymerase. 25 µM α - 32 P-UTP (400 Ci/mmol) was used for labelling the probe. The transcription mix was treated with

RNase-free DNaseI (Boehringer) for 10 minutes at 37°C, phenol extracted and ethanol precipitated. The RNA pellet was redissolved in 80% Formamide, 1xTBE, loaded on a 5% polyacrylamide/8M urea gel and the full length transcript was isolated by electroelution.

RNase Mapping

Hybridization and RNase mapping were performed essentially as described by Melton et al. (30). 50 µg of total cellular RNA were hybridized overnight with 1.5x10⁵ cpm of RNA probe at 42°C. RNase digestion took place in the presence of 50 units/ml RNase T1 (Boehringer) and 3 mg/ml RNase A (Boehringer) at 37°C.

RESULTS

Negative and Positive Elements Control Lysozyme Gene Expression in a Chicken Macrophage Cell Line

To test the expression of transfected lysozyme gene recombinants we chose the macrophage cell line HD11. This is an established line of chicken macrophages transformed by the myc-containing MC 29 virus (23,31). The activity of the lysozyme gene is about one third of that detected in primary cultures of mature macrophages (2,16). Plasmids containing 5' deletions of the chicken lysozyme upstream region fused to the CAT reporter gene were

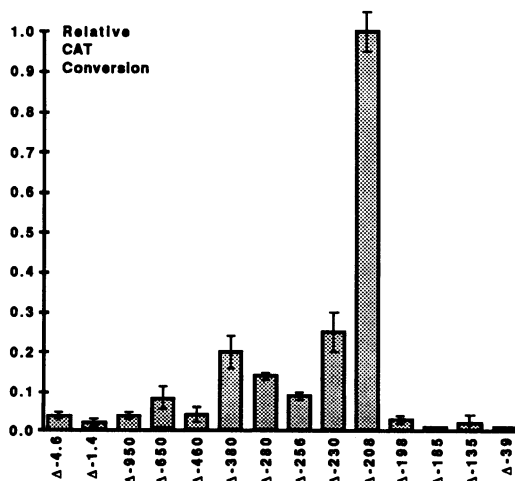


Figure 1. CAT expression of the lysCAT 5' deletion series in the macrophage cell line HD11. Transient expression from each of the plysCAT Δ -n plasmids was determined in triplicate experiments and expressed relative to the activity of plysCAT-208. Standard deviations are indicated.

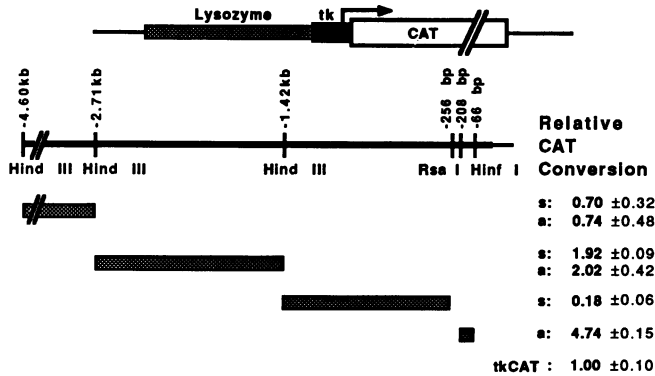


Figure 2. Dissection of 4.6kb upstream sequences into four segments. Four lysozyme upstream sequences were cloned in front of the tkCAT fusion gene and transfected into HD11 cells. Schematic representation of lysozyme constructs in sense (s) or antisense (a) orientation are shown. Mean values of CAT activities with their standard deviations are expressed relative to the activity measured with pBL-CAT2 (tkCAT).

transfected into HD11 cells, which were then analyzed for CAT activity (figure 1). The amount of CAT activity determined is markedly dependent on the deletion mutant used: mutant -208bp shows the highest expression, whereas further deletion of 10bp (mutant -198bp) results in only background activity. Maintaining 230bp or more upstream of the start site leads to a suppression of the CAT activity. This highly reproducible patterns of increase and subsequent decrease in CAT expression with the inclusion of progressively more 5' sequences indicated that lysozyme gene activity may be controlled by both positively and negatively acting elements. Since it is difficult to analyze such an organization with deletion mutants, we divided 4.6kb of lysozyme upstream sequences into 4 fragments. These fragments were cloned in front of the herpes simplex thymidine kinase promoter (tk) of the vector pBL-CAT2 (gift of B. Luckow and G. Schütz). The CAT expression from these recombinants in HD11 cells (figure 2) showed some enhancer activity from lysozyme sequences -2.71kb/-1.42kb and -208bp/-66bp, even in the anti-sense orientation. A negative effect was observed with lysozyme fragment -1.42kb/-253bp. Since we wanted to focus first on the enhancer sequences, the fragment -1.42kb/-253bp was not further analyzed.

Two Enhancer Elements Can be Identified which are Multiplicative in their Effects

Based on the results shown in figure 2 we sub-divided the long -2.71kb/-1.42kb fragment into small segments. Analysis of the effects of these sequences on CAT expression revealed enhancer activity of a fragment from -2.71kb to -2.54kb (figure 3B). Hence at least two enhancer elements, each of about 150bp, were found to be active in HD11 cells. These two enhancer elements, E-2.7kb (lys -2.71kb/-2.54kb) and E-0.2kb (lys -208bp/-66bp),

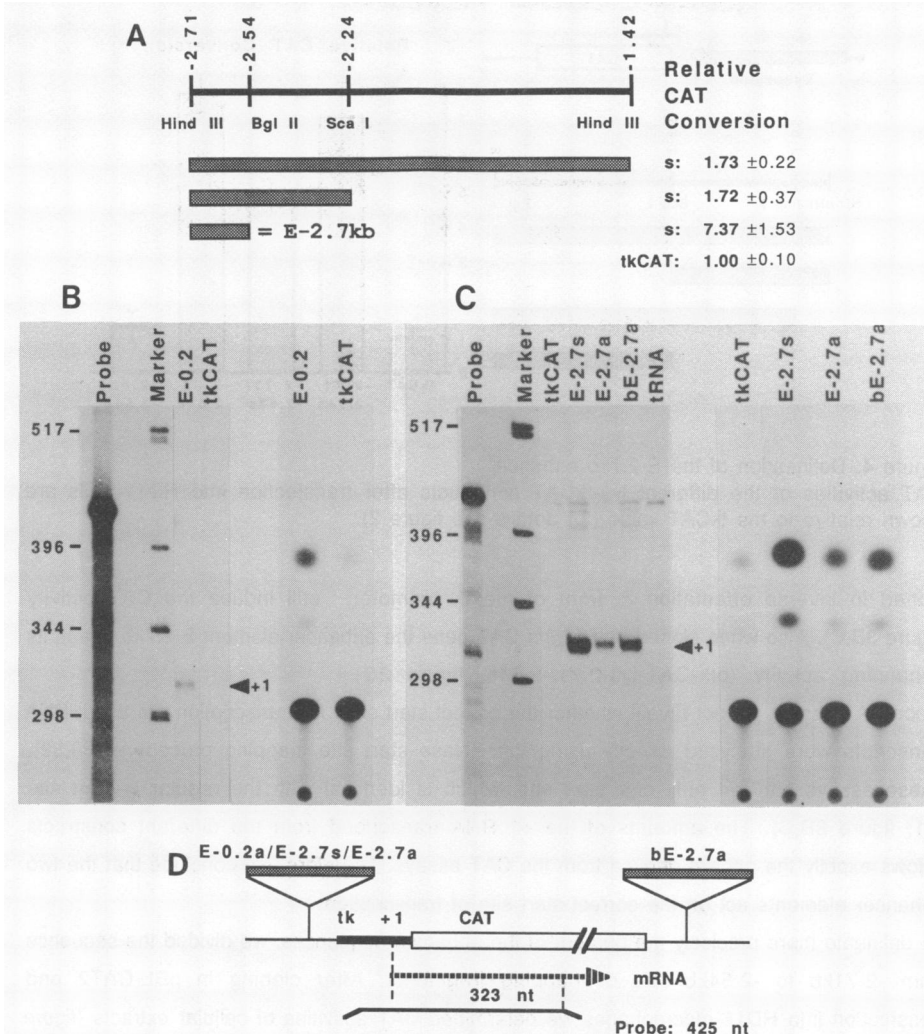


Figure 3. Enhancer activities of the E-0.2kb and E-2.7kb fragments act on correctly initiated RNA.

CAT assay and RNA start site mapping experiments were performed on HD11 cells after transfection with lys-tkCAT constructs containing the E-0.2kb or the E-2.7kb enhancer sequences. **A** Diagram of lysozyme upstream sequences cloned in front of tkCAT with their CAT activities shown. **B** RNA transcript mapping and CAT activities of pLys-208/66a tkCAT (E-0.2a) and pBL-CAT2 (tkCAT). In addition to the +1 start site the size of the labelled RNA probe (Probe) and *Hinf*I digested end labelled pBR322 (Marker) are shown. A thin layer chromatogram exhibits the amounts of ^{14}C -chloramphenicol (lower spot) and of the acetylated forms (upper spots). **C** RNA transcript mapping and CAT activities of E-2.7s, E-2.7a and bE-2.7. **D** Diagram depicting the different constructs used and the length of the RNase protected RNA fragment.

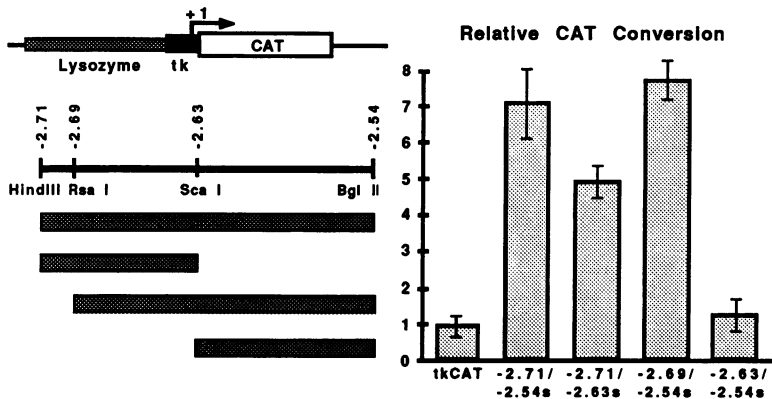


Figure 4. Delineation of the E-2.7kb enhancer.

CAT activities of the different lys-tkCAT constructs after transfection into HD11 cells are shown relative to the tkCAT value (for details see figure 2).

cloned in inverse orientation in front of the tk promoter, still induce the CAT activity (figure 3B,C). Also when cloned behind the CAT gene the enhancer element E-2.7kb exerts its enhancing activity (ptk-CAT-lys-2.71/-2.54s, figure 3C).

Since CAT assays do not reveal whether the correct start sites for transcription are used, RNA transcripts were analyzed directly using the RNase start site mapping procedure (30,32). These assays showed only one start site, which is identical with the regular tk-start site (+1; figure 3B,C). The amounts of the +1 RNA transcribed from the different constructs follows exactly the pattern derived from the CAT assays. Therefore, we conclude that the two enhancer elements act on the correct start site of transcription.

To delineate more precisely the borders of the enhancer sequences, we divided the sequence from -2.71kb to -2.54kb into overlapping fragments. After cloning in pBL-CAT2 and transfection into HD11 macrophages we determined CAT activities of cellular extracts (figure 4). Since lys-2.69/-2.54 shows full enhancer activity, while lys-2.71/-2.63 retains some activity, and lys -2.63/-2.54 is inactive, the 5'border of the enhancer can be positioned between -2.69kb and -2.63kb and the 3'border between -2.63kb and -2.54kb. The 5'border of the E-0.2kb enhancer is defined by the two deletion mutants -208bp and -198bp (figure 1). The 3'end of the enhancer fragment (-66bp) can not be shortened without loss of enhancing activity (not shown). Since we determined the enhancer activity of the individual E-2.7kb and E-0.2kb fragments, we wanted to measure their combined effects on the lysozyme promoter. The basal activity of the lysozyme promoter is determined from the deletion mutant -198bp. Addition of 10bp restores the E-0.2kb enhancer activity which stimulates transcription about 30-fold (figure 5). The addition of the E-2.7kb enhancer

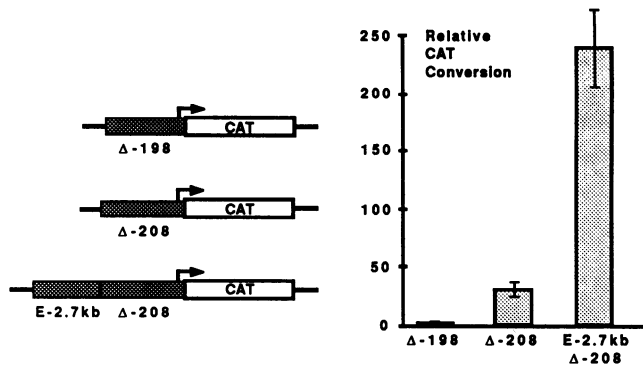


Figure 5. Multiplicative action of the combined enhancer sequences E-0.2kb and E-2.7kb. Plasmids *plysCAT*Δ-208 and E-2.7Δ-208 contain the E-0.2kb and the E-0.2kb plus the E-2.7kb enhancer, respectively. CAT activities of these plasmids after transfection into HD11 cells are expressed relative to the activity found with the enhancerless construct *plysCAT*Δ-198.

leads to about 200-fold induction proving the multiplicative effects of the combined enhancers.

A Negative Element is Located Immediately Downstream of the E-2.7kb Enhancer

In our attempt to characterize the E-2.7kb enhancer, we found different degrees of enhancing activity depending on the presence of sequences downstream of the E-2.7kb enhancer (figure 3A). To analyze this phenomenon, we cloned three overlapping fragments into pBL-CAT 2: *lys* -2.71/-2.25, *lys* -2.71/-2.54 and *lys* -2.54/-2.25. After transient expression in HD11 cells we determined a high CAT activity for the E-2.7kb enhancer, a reduced enhancing activity after testing a longer fragment containing an additional sequence (-2.54/-2.25) and even an inhibition of the tk promoter activity by the sequence -2.54/-2.25 alone, independent of its orientation (figure 6A). Further deletion into this fragment weakens the negative effect.

This evidence was again confirmed using the RNase start site mapping procedure, which revealed a 2-3 fold lower enhancement by *lys*-2.71/-2.25 than by *lys* -2.71/-2.54 (figure 6). The effect of the negative element on the tk promoter without enhancer sequence could not be measured by the RNase mapping technique since the expression of this construct is reduced below the detection limits of this assay.

These data show that the negative element (N-2.4kb), independent of its orientation, can reduce tk promoter activity both in the presence or absence of the E-2.7kb enhancer element. We hybridized N-2.4kb DNA to a "Southern" blot of chicken genomic DNA and found that this fragment belongs to the class of unique sequences (not shown).

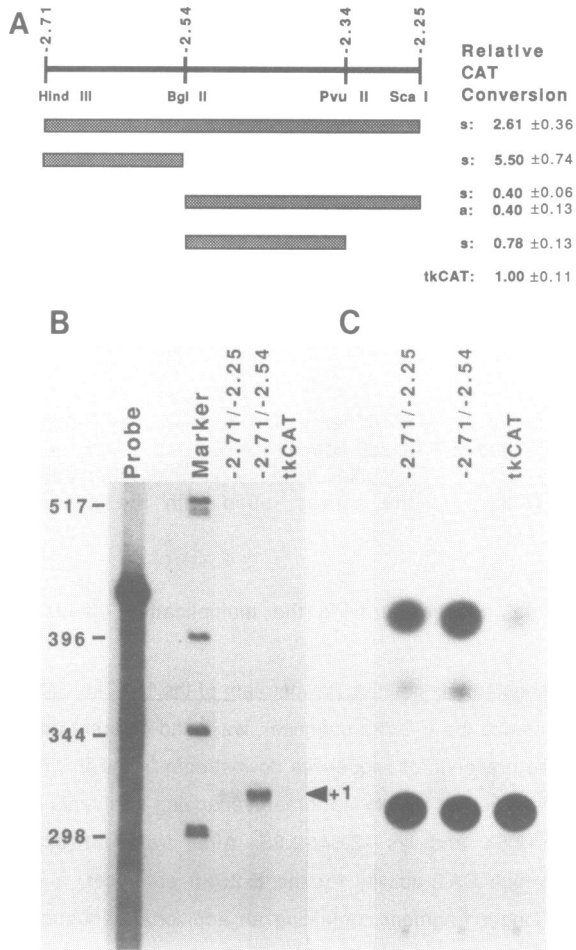


Figure 6. The lysozyme upstream sequence -2.54kb to -2.25kb (N-2.4kb) decreases transcription.

Different lysozyme sequences cloned in front of the tkCAT fusion gene were transfected into HD11 cells and assayed for their CAT activity and RNA start site. **A** Schematic representation of lysozyme constructs and their CAT activities in sense (s) or antisense (a) orientation. **B** RNA start site mapping experiment and **C** thin layer chromatogram of the CAT assay as shown in figure 3.

Both Positive and the Negative Elements Act in a Tissue Specific Manner

Our aim was to analyze the macrophage specific expression of the chicken lysozyme gene. Therefore, we wanted to test the activity of the two enhancer elements and of the negative element in different cell types. In addition to the chicken myeloid cell line HD11 (not fully differentiated macrophages) we used primary mature macrophages and a chicken embryo

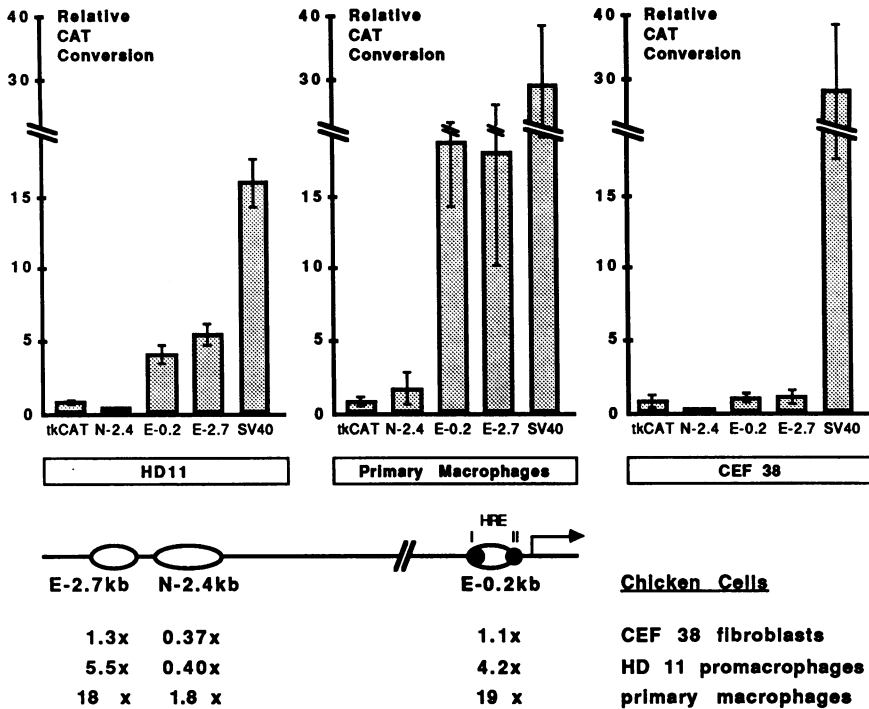


Figure 7. Cell specificity of the enhancer elements and of the negative element. The fibroblast cell line CEF38, the promacrophage line HD11 and primary mature macrophages were transfected with plys-2.54/-2.25s tkCAT (N-2.4), plys-208/-66a tkCAT (E-0.2), plys-2.71/-2.54s tkCAT (E-2.7) and as control pBL-CAT2 (tkCAT) and pSVtkCAT (SV40) CAT values are expressed relative to the activity measured from pBL-CAT2. The diagram and table at the bottom of the figure depict the positions of the three elements and their effects in the different cell types tested. HRE I and II are the two hormone responsive elements (4,5).

fibroblast cell line CEF 38. Both enhancer elements are active in the macrophage cell line, somewhat more active in the primary mature macrophages, but are inactive in the fibroblast line (figure 7). These elements, therefore, seem to contribute tissue specifically to the lysozyme expression in the macrophage cells. The negative element lys-2.54/-2.25 represses transcription from the tk-promoter in fibroblast and the myeloid cell line, but is neutral in the primary mature macrophages (figure 7).

Sequence Comparisons

The two enhancer sequences E-2.7kb and E-0.2kb were compared with each other and with other known enhancer sequences. Comparison with each other revealed a sequence element, which was found once in E-2.7kb and twice in E-0.2kb ("lys" in figure 8A and B) and shows

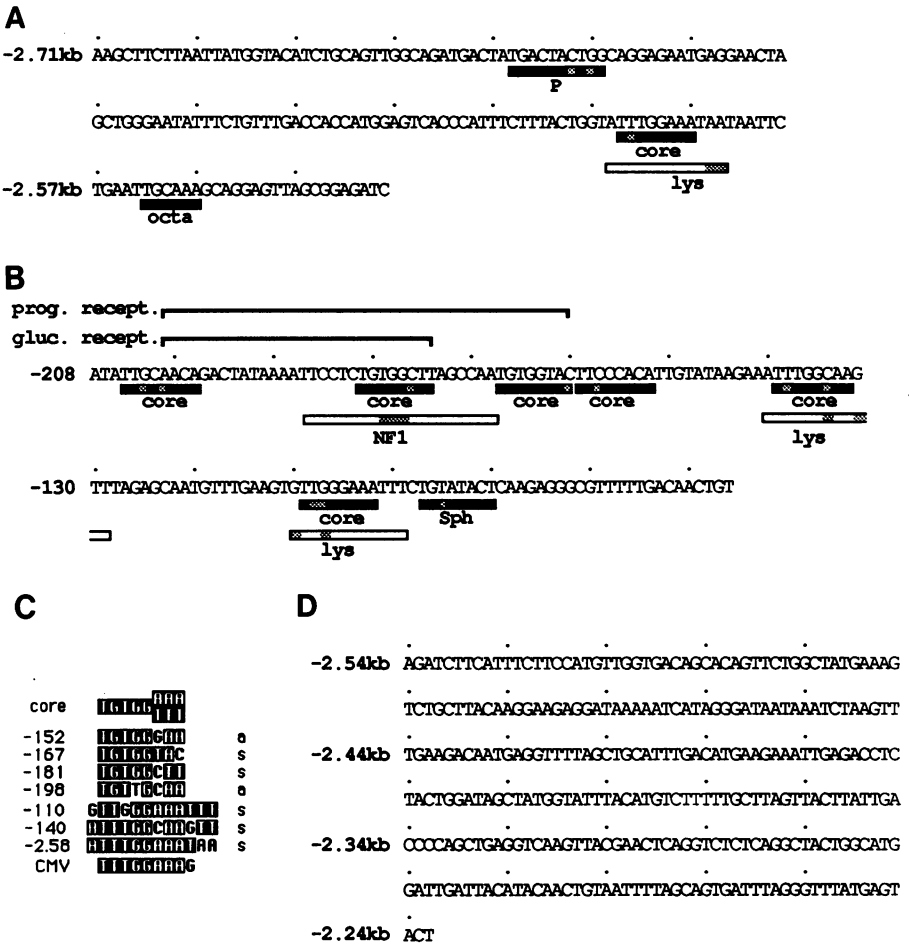


Figure 8. Sequence analysis of the three lysozyme regulatory elements.

A The E-2.7kb enhancer fragment contains sequences homologous to the SV40 p (p) and core (core) motifs (33,36), to the Ig octamer (octa) sequence (8,37) and to the lysozyme enhancer E-0.2kb (lys). The filled or open bars indicate the length of the homologous sequence, shaded areas indicate non-matching nucleotides. **B** The E-0.2kb enhancer element shows homologies to the SV40 core (core) and Sph motif (Sph) (33,36), to the nuclear factor 1 (NF1) binding site (38) and two sequences homologous to each other and to the lysozyme enhancer E-2.7kb (lys). **C** All "core" and "lys" homologies are compiled and compared to the cytomegalovirus enhancer (CMV) (37). **D** The sequence of the N-2.4kb element.

homology to the SV40 enhancer core sequence (33,34) as well as to the CMV enhancer (35). This homology is pointed out in figure 8C. Further comparison of E-2.7kb and E-0.2kb with known enhancer sequences revealed more homologies to the SV40 core sequence: six

homologous regions in E-0.2kb and one in E-2.7kb. One homology to the Sph element of the SV40 enhancer domain A (36) was found in E-0.2kb and one homology to the P motif of the SV40 enhancer domain A was detected in E-2.7kb. One sequence element homologous to the octamer motive (Ig gene; 8,37) and one homologous to the NF1 binding site (38) were identified in E-2.7kb and E-0.2kb, respectively (figure 8A and B). In addition to these homologies the enhancer E-0.2kb contains binding sites for both the glucocorticoid receptor and the progesterone receptors (4,5).

DISCUSSION

By analyzing upstream sequences of the chicken lysozyme gene with transient DNA transfer experiments in chicken macrophage cells we have identified three distinct regulatory sequences. Two elements act as macrophage specific enhancer elements and one is a negative element, which is inactive in mature macrophages.

Other tissue specific genes have also been found to be regulated by tissue specific enhancer sequences (for review see (39)). In several of these cases sequence homologies or protein binding experiments have shown that some of the components which build up a tissue specific enhancer can also be found in enhancer elements of different specificity or in combination with promoter or replication function. E.g. the octamer motif within the lymphocyte specific immunoglobulin enhancer (6,7) is also found upstream of many other genes (40). Nuclear factor I, a protein required for adenovirus replication (41) has been shown to be an ubiquitous factor, which binds to several enhancer sequences (for review see (42)) and to the CCAAT promoter element (43); it also binds to a 579 bp lysozyme fragment, which acts as a macrophage specific enhancer element (16). The macrophage specific enhancer E-0.2kb also contains a significant homology to the NF1 binding consensus sequence, whereas the enhancer sequence E-2.7kb does not. Several homologies to the SV40 enhancer can be seen in both these enhancer sequences, as well as in the IgH enhancer (6,7,36), CMV enhancer (35), adenovirus 2 E1a enhancer (44) and the c-fos enhancer (45).

Multiple homologies both between lysozyme enhancer sequences (E-2.7kb and E-0.2kb) and with other enhancer elements suggest a complex modular structure. This is emphasized by the fact that the footprint region of the progesterone and glucocorticoid receptors (4,5) within the E-0.2kb element can not be deleted without destroying enhancer activity since 5' deletion mutant -164bp loses enhancer activity as well as steroid regulation in oviduct cells (4). The observed enhancer activity in macrophages is not due to a steroid induction, since the use of antihormones did not influence enhancer activity (not shown). This may indicate that steroid receptors and enhancer binding factors can recognize similar DNA sequences. Such a possibility has been discussed recently, since the DNA binding domains of steroid receptors,

the thyroid receptor and the viral erbA product are quite homologous (for review see: (46)). In the chicken ovalbumin gene sequences required for steroid regulation have also been found to overlap with another upstream regulatory region, which is in this case a 'blocker' sequence (47).

For the chicken lysozyme gene we identified a negative element in the immediate vicinity of an enhancer sequence. This negative element at -2.4kb upstream of the start site suppresses enhancer function of the E-2.7kb enhancer in an incompletely differentiated macrophage line, but is inactive and therefore allows complete enhancer activity in mature macrophages. Cell specific negative elements have also been found to regulate expression of other genes: rat growth hormone gene (48), rat α -fetoprotein gene (12), rat insulin 1 gene (49) and the IgH gene (50), and in some cases appear to be closely associated with an enhancer sequence.

In several cases, DNase I hypersensitive sites of the chromatin reflect positions for regulatory sequences. All three identified lysozyme regulatory sequences, E-2.7kb, N-2.4kb and E-0.2kb are located in regions of previously determined hypersensitive sites (HS) (51). Appearance and disappearance of these sites follows exactly the observed activities of the three elements: HS-2.7 and HS-0.1kb are present in macrophages, but are absent in fibroblasts, whereas HS-2.4kb is absent in mature macrophages, the only cell type in which we find the negative element N-2.4kb to be neutral. Based on this correlation, and on the tissue specific effect of the negative element, we conclude that N-2.4kb as well as E-2.7kb and E-0.2kb probably interact with cellular factors.

The additive effect of all the regulatory elements described here, together with the enhancer at -6.1kb (16) and possibly together with as yet unidentified sequence elements, probably activate the chicken lysozyme gene during the differentiation of macrophages. A similar regulation by a complex array of positive and negative regulatory regions has been found for the rat and mouse α -fetoprotein genes (12,13). Since the precise arrangement of these elements does not appear to be of absolute importance to their function, the multitude of different regulatory regions might have served during evolution to modulate both the magnitude and the tissue specificity of gene activity.

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