Cooperative interaction of chicken lysozyme enhancer sub-domains partially overlapping with a steroid receptor binding site

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ABSTRACT

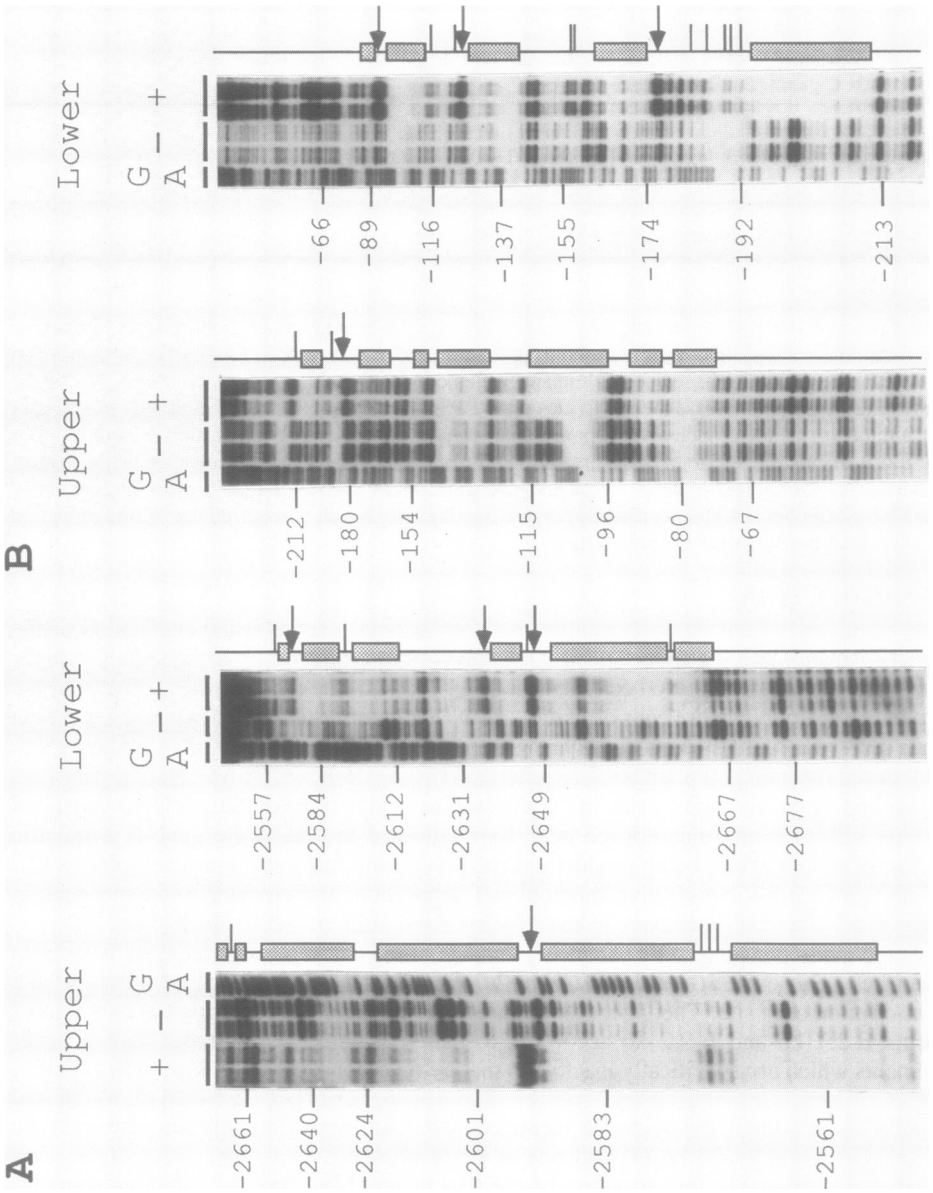
Expression of the lysozyme gene is a marker for the differentiation of macrophages, lysozyme transcription being gradually increased during maturation. We have analyzed the fine structure and function of two macrophage-specific enhancer elements of the chicken lysozyme gene (E-2.7 kb and E-0.2 kb). Both increase their activities upon LPS induction, both contain multiple binding sites for similar or identical nuclear factors and both can be divided into two functional modules. For the E-0.2 kb enhancer we found a synergistic activity of the modules to be dependent on their distance. Binding sites for nuclear proteins within enhancer E-0.2kb overlap substantially with the previously identified progesterone/glucocorticoid receptor binding site, which is required for steroid induction of lysozyme transcription in the oviduct.

INTRODUCTION

One of the main contemporary challenges in molecular genetics is the elucidation of the mechanisms underlying tissue specific gene expression. Several tissue specific promoter and enhancer elements have been identified (1). Many of the model systems analyzed are limited in that only mature cells or tissues are available for testing, whereas gene regulation may involve a multi step process during differentiation from precursor to mature cells. The haematopoetic cell population is generated by one of the few differentation pathways which can be studied step by step in vitro as well as in vivo. One of the cell types generated in this system is the macrophage cell. Expression of the lysozyme gene is a specific differentiation marker, being gradually turned on during maturation of macrophages (2). In addition to this constitutive macrophage expression, the chicken lysozyme gene is inducible by steroid hormones in the tubular gland cells of the oviduct (3).

Three macrophage-specific enhancer elements of the chicken lysozyme gene have been identified, one each at −6.1 kb (4), −2.7 kb (5) and −0.2 kb (5) upstream of the transcription start site. The latter two elements are referred to here using the abbreviated forms E-2.7 kb and E-0.2 kb according to their positions. Furthermore, two silencer elements which are specifically inactive in mature macrophages (S-2.4 kb and S-0.25 kb), and one which is active in all cell types tested (S-1.0 kb) have been described (5, 6). Steroid induced expression in the oviduct is mediated by at least two different hormone responsive elements, one at −200 bp (7) and one at −1.9 kb (8).

Here we report the fine structure of the two macrophage specific enhancer elements E-2.7 kb and E-0.2 kb, their functional activity and their capacity to bind nuclear proteins. The binding pattern in the multifunctional enhancer E-0.2 kb, which also contains functional steroid receptor binding sites (7), suggests a competition between steroid receptors and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-2601
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-2557
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-2631
-2649
-2667
-2677
B
Upper
G − + A
-212
-180
-154
-115
-96
-80
-67
Lower
G − + A
-66
-89
-116
-137
-155
-174
-192
-213

enhancer factors allowing steroid induction in the oviduct and constitutive expression in macrophages.

MATERIALS AND METHODS

Plasmid Constructions

The identity of the recombinant plasmids was confirmed by restriction mapping and dideoxy-sequencing using the Sequenase-kit from USB.

The plasmids pICfrag21 and pUClys−280/+15 were created by inserting the HindIII/BglII-lysozyme gene fragment from −2.71 kb to −2.54 kb and the HindIII/XhoI-fragment carrying the lysozyme-promoter (−280 to +15) from plysCATΔ−280 (5) into the corresponding sites in the polylinker of pIC20R (9).

Two series of different plasmids containing subregions of lys E-0.2 kb were used, all of which are derivatives of the plamids pBLCAT2 (10) or plys−208/−67a tkCAT (5). One series contains a spacer of varying length between lys −67/−161 and lys −162/−208 in front of tkCAT. This series was constructed by introducing a 20 bp synthetic oligonucleotide containing recognition sites for SnaBI, StuI, NruI and SalI into the natural RsaI-site in position −161 in plys−208/−67a tkCAT, and performing subsequent deletions using these endonucleases. Plasmids with spacer-lengths of 5, 8, 10, 13, 15, 17 and 20 bp were constructed.

The second series contains various combinations of the lysozyme fragments from −208 to −162 and −161 to −67 in front of the tkCAT-fusion gene. These plasmids were constructed from a subclone of lys −67/−208 in pUC 18, of lys −95/−208 in pUC 18, of plysCATΔ208 and pBLCAT2 (10). Selected lysozyme sequences were inserted in antisense direction in front of the tk-promoter to test them directly for enhancer-characteristics.

pE-0.2−1 was generated by ligating the lysozyme BamHI/AccI fragment from pUC lys −208/−95 into the BamHI site of pBLCAT2.

pE-0.2−2 contains the lysozyme sequences from −208 to −162 as an RsaI/BamHI-fragment cloned into the BamHI and filled-in SalI sites of pBLCAT2.

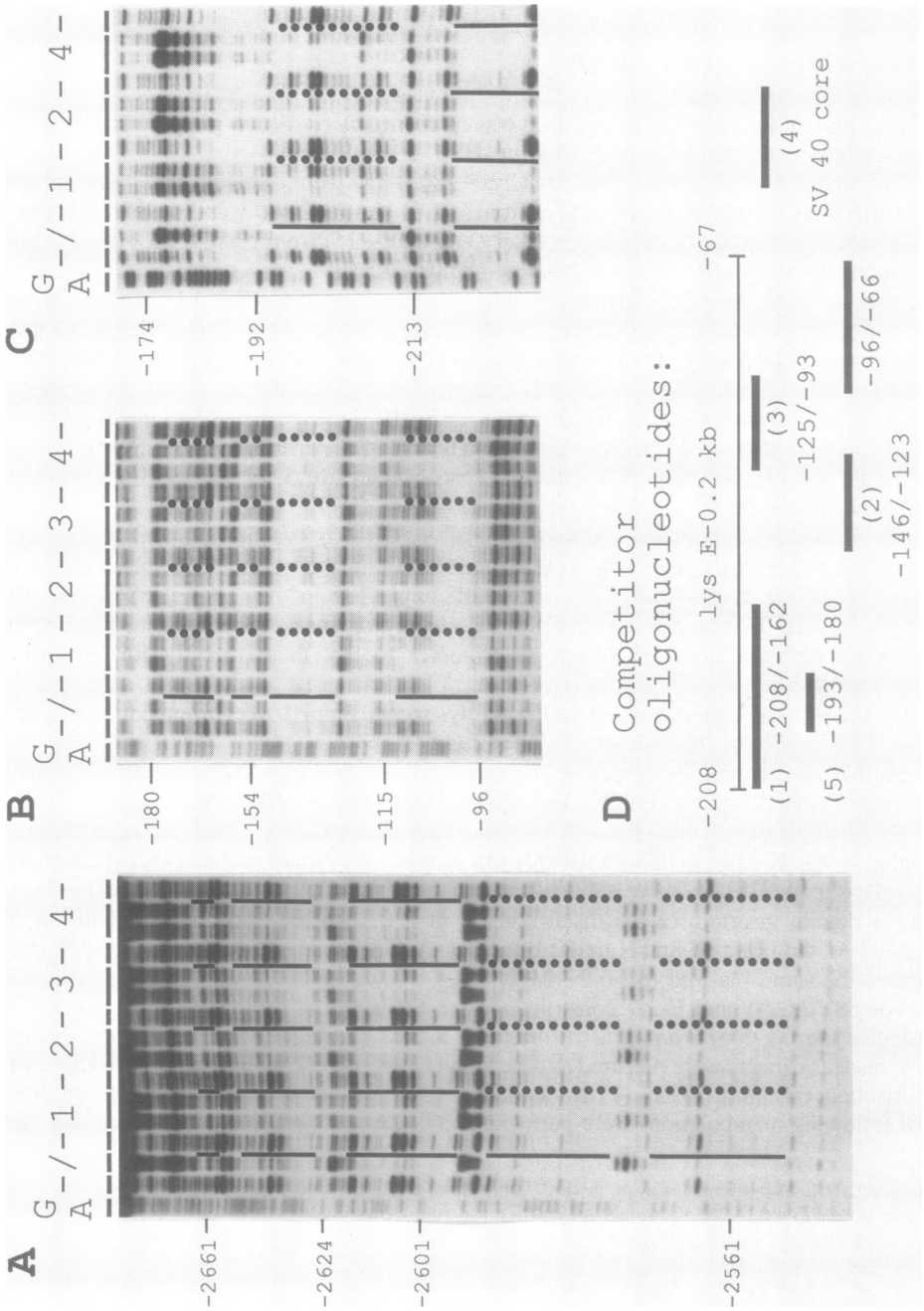
By cloning a blunt-ended HindIII/RsaI-fragment from pUClys−208/−67 harboring the region from −161 to −67 into the filled in BamHI- or HindIII-site of pBLCAT2 or the BamHI-site of pE-0.2−2, we obtained the plasmids pE-0.2−3, −4, −5, −6 and −2×3. For a double insertion of the distal domain of E-0.2 kb this sequence was cut out of pE-0.2−2 with BamHI and HindIII, filled in and re-inserted into the blunt-ended HindIII site of the same plasmid (pE-0.2−2×2).

For pSVtkCAT and E-2.7 constructions see Steiner et al., 1987 (5).

Nuclear Extract Preparation

The nuclear extracts were prepared as described (11−13). Subconfluent cells were harvested from about 60 culture dishes (15 cm in diameter) and washed in phosphate buffered saline. All following manipulations were performed at 0°C using precooled solutions, tubes and

Figure 1. DNaseI protection assay on the E-2.7 kb (A) and the E-0.2 kb enhancer (B). The upper (upper) and lower strands (lower) were 3′ end-labelled and digested with DNaseI in the presence of HD11 nuclear extract (+) or without nuclear extract (−). The G+A sequencing products are shown as molecular weight markers. The shaded diagram on the right depicts the footprinting pattern achieved, boxes indicate protected sequences, arrows show positions of strong DNaseI hypersensitivity caused by nuclear extracts and horizontal lines poin to weak hypersensitive sites.


A
G
A
-/-1-2-3-4-
-2661
-2624
-2601
-2561
B
G
A
-/-1-2-3-4-
-180
-154
-115
-96
C
G
A
-/-1-2-4-
-174
-192
-213
D
Competitor
oligonucleotides:
-208
lys E-0.2 kb
-67
(1) -208/-162
(3)
-125/-93
(4)
SV 40 core
(5) -193/-180
(2)
-146/-123
-96/-66

centrifuges. The pelleted cells were suspended in five packed cell volumes (PCV) of buffer A′ (10 mM Hepes/KOH (pH 7.6), 10 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1mM EDTA, 0.5 mM DTT) and allowed to stand on ice for 10 min. The cells were pelleted by centrifugation and suspended in 2 PCV. The cells were broken by homogenization in a glass-glass dounce homogenizer. After addition of one-tenth volume of buffer B′ (10mM Hepes-KOH (pH 7.6), 1M KCl, 0.15 mM spermin, 0.5 mM spermidin, 1mM EDTA, 0.5 mM DTT) the nuclei were pelleted by centrifugation (2400 rpm; 10 min). This pellet was used to prepare the nuclear extract as described (12) with the modification that the volume of nuclear lysis buffer was 1 PCV and that the precipitated nuclear proteins were dissolved in 1/10 PCV of the dialysis buffer. The opaque nuclear extract with a protein concentration of 5−20 mg/ml (Biorad protein assay) was frozen in small aliquots in liquid nitrogen and stored at −85°C.

DNaseI-Footprinting and Band Shift Assays

To obtain probes labelled on one strand the plasmids pICfrag21 and pUClys−280/+15 were cut with EcoRI and labelled as described (14). The footprint reactions were performed according to Cereghini et al., 1987 (15) with the described modifications (14). The following blunt ended, double stranded oligonucleotides were used: lysozyme sequences −208/−162, −193/−180, −146/−123, −125/−93, −96/−66; SV40 core: GTTAGGGTGT-GGAAAGTCCCCAG; SP1 SV: CGACTGATCAGTTCCGCCCATTCTCCGCCCCAG (with AccI/BamHI sticky ends); Sp1 dhfr: CTTGGTGGGGGCGGGGCCTAAGCTG (16); AP4: GATCACCAGCTGTGGAAT (17).

Band shift assays were performed in a total volume of 10 µl containing: 250 ng poly(dIdC) (Pharmacia), 25 ng denatured calf thymus DNA, 5% glycerol; 25 mM Hepes/KOH pH = 7.6, 25 mM KCl, 5 mM $MgCl_2$, 0.4 µg nuclear extract and ^{32}P-labelled double stranded oligonucleotide (30,000 to 40,000 cpm). The nuclear extract was preincubated for 15 min on ice before addition of the labelled probe. After further 20 min incubation on ice the samples were loaded directly on a 5% acrylamide gel (acrylamide:bisacrylamide ratio of 79:1 in 192 mM glycin / 25 mM Tris buffer).

The gel was run for 1.5 hrs at 15 to 20 mA at +4°C, fixed, dried and autoradiographed.

Cell Culture, Transfection and CAT-Assays

HD11 [=HBC1=LSCC-HD(MC/MA1) (18)] and DU249 (19) cells were maintained in DME-medium (Biochrom) supplemented with 8% fetal calf serum, 2% chicken serum, 100 units/ml penicillin and 100 µg/ml streptomycin. All transfection experiments were done in triplicate and confirmed in a second series of tests. Transfections were carried out as described (5) with the help of an automated 'precipitator' (20). LPS treatment of HD11 cells was done by applying 0.1 µg/ml LPS to the dishes at 22hrs after transfer. All cells were harvested 2d after DNA transfer and assayed for CAT activity or for mRNA amounts (5).

Figure 2. Competitions of the footprints on the enhancer E-2.7 kb and E-0.2 kb. Footprint reactions without nuclear extract (−) and with HD11 nuclear extract either without competitor (/) or with double stranded oligonucleotides (1), (2), (3) or (4) in amounts of 0.5 pmole (left lanes) or 2.5 pmole (right lanes). Vertical lines indicate the positions of protected regions, whereas the dots point out competed footprints. The following 3′ end-labelled DNA fragments were used: upper strand of E-2.7 kb (A), upper strand of E-0.2 kb (B) and, for better resolution, lower strand of E-0.2 kb (C). Panel D depicts the different oligonucleotides used.

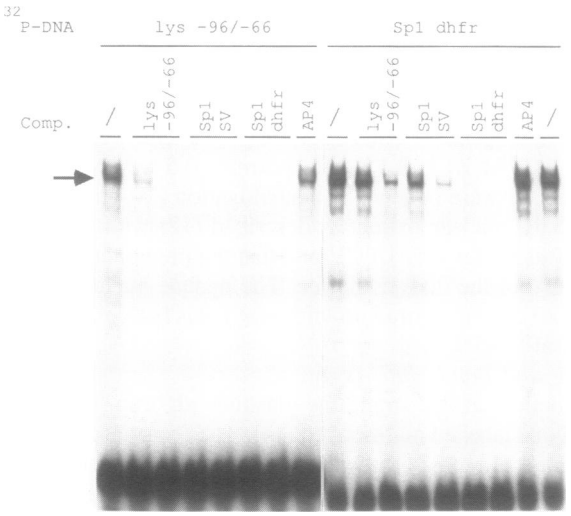


Figure 3. Gel shift experiments with lysozyme (lys −96/ −66) and Sp1 (Sp1 dhfr) oligonucleotides. HD11 nuclear extract was assayed for specific DNA binding activity. Competition experiments were done using two different concentrations (0.1 pmole and 1pmole in the first and second lane, respectively) of double stranded oligonucleotides lys −96/−66, Sp1 SV and Sp1 dhfr. The AP4 oligonucleotide was used at a concentration of 1 pmole. The arrow points to the major retarded band.

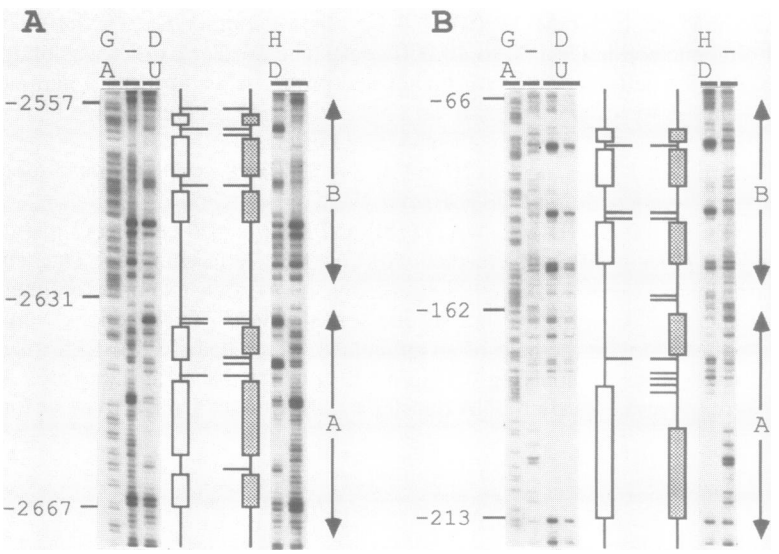


Figure 4. Comparison of HD11 and DU249 nuclear extracts in footprint reactions. The lower strand of E-2.7 kb (A) and the lower strand of E-0.2 kb (B) were 3′ end-labelled and incubated with HD11 (HD) or DU249 extract (DU) and DNase I digested. For comparison footprint reactions without nuclear extract (−) and G+A sequencing products (GA) are shown. Strong and weak DNase I hypersensitive sites are pointed out by horizontal lines.

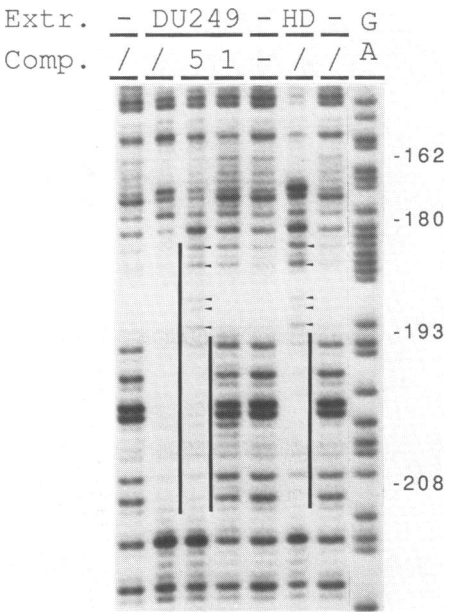


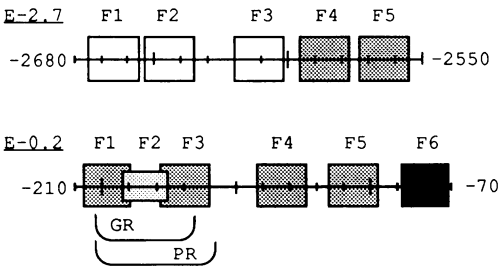
Figure 5. Competition of the liver cells specific footprint on the E-0.2 kb enhancer. Footprint reactions with DU249 (DU), with HD11 (HD) or without (−) nuclear extract are shown. Competitions with unlabelled double stranded oligonucleotide 1 or 5 (−208/−162 or −193/−180 lysozyme sequences; see Figure 2D) were carried out with 3−10 pmole as competitor DNA [(1) and (5); (/) is without competitor DNA]. Vertical lines indicate the footprint regions and the arrow heads point to hypersensitive sites.

RESULTS

Several Factors Bind to Both Enhancers E-2.7 kb and E-0.2 kb

We have previously identified two macrophage specific enhancer elements upstream of the chicken lysozyme gene (5). Sequences of about 150 bp each are required for optimal function of both enhancer sequences. Since the size of these elements suggested the possible presence of several binding sites for nuclear proteins, we carried out footprinting experiments using total nuclear extracts. Nuclear extracts were purified from a chicken promacrophage cell line (HD 11) in which both enhancer elements are functional (5).

Footprinting experiments on both enhancer sequences demonstrated the protection from DNase I digestion of multiple regions (figure 1). Sequence analysis of both enhancer elements has revealed a common sequence motif present several times in E-2.7 kb and in E-0.2 kb (5). This motif is similar to the SV40 enhancer core sequence (21). Since most of these sequence elements are protected from DNase I digestion in the footprinting experiments (see figure 6), we tested for relationship between the bound proteins. We used four different oligonucleotides to compete the protein binding in footprint reactions (figure 2). Oligonucleotide [1] contains lysozyme sequences from −208 bp to −162 bp including two core motif homologies; oligonucleotides [2] and [3], which correspond to the regions −146 bp to −123 bp and −125 bp to −93 bp, respectively, contain one core motif each. The oligonucleotide [4] contains the original SV40 core sequence [SV40 sequences 233 to 255; BBB system (22)]. The footprint competitions show that the proteins bound to the core motif homologies can be competed with each of the four oligonucleotides



	Extract M	Extract L	Lysozyme Sequence / Similarity	Identity	Reference
E-2.7					
F1	+	+	TGACTATGACTACTGGC		
			TGACTA	GCN4 - AP1	24
			TGACTA	GCN4 - AP1	24
F2	+	+	AGGAGAATGAGGAACTAGC		
			GAAaGAGGAACT	SV40 PU-box	25
F3	+	+	GACCACCATGGAGTCACCCA		
			GAGTCA	GCN4 - AP1	24
F4	+	+	ACTGGTATTTGGAAATAATAA		
			TTTGGAAAT	lys. cons.	5
			TgTGG$^{AAA}_{ttt}$T	AP3 - C/EBP	28
F5	+	+	TCTGAATTGCAAAGC		
			aTGCAAAt	octa	29,30
			A$^{Aaa}_{tTT}$cCAcA	AP3 - C/EBP	28
E-0.2					
F1	+	+	GATATTGCAACAGACTA		
			A$^{aaa}_{TTt}$CcACA	AP3 - C/EBP	28
F2	-	+	TATAAAATTCCTCTG		
			AAATTCCTCTG	NRD I	32
F3	+	-	TGTGGCTTAGCCAATGTGGT		
			TTAcaCAATGTGGT	C/EBP	31
			TGTGG$^{aaa}_{tTT}$g	AP3 - C/EBP	26,28
F4	+	+	AGAAATTTGGCAAGTTTAG		
			TTTGGCAAG	NF1	15
			TTTGGaAAt	lys. cons.	5
			TgTGG$^{aAA}_{ttt}$G	AP3 - C/EBP	26,28
F5	+	+	GAAGTGTTGGGAAATTTCTG		
			TTtGGAAAT	lys. cons.	5
			TgtGG$^{AAA}_{ttt}$g	AP3 - C/EBP	26,28
F6	+	+	CAAGAGGGCGTTTTTG		
			GAGGGCGT	Sp1	33

(figure 2). Although differences in binding efficiences are seen—each oligonucleotide showing the best competition for the footprint on its corresponding region—it is obvious that all the 'core'-containing sequences show qualitatively identical competitor function in comparison to footprints which are not changed, such as the footprint upstream of −217 bp (figure 2C), which lies within the silencer element S-0.25 kb (6) and the E-2.7 kb footprints outside the 'core' sequence-containing region (E-2.7 kb sequences −2600 bp to −2700 bp; figure 2A).

One of the footprint regions (sequences −90 to −74) in the E-0.2 kb enhancer could only be detected quite weakly, and since the sequence resembles a Sp1 binding site (see figure 6), we performed band shift experiments with lysozyme DNA −96/−66 (figure 3). One major shifted band can be seen in addition to several minor bands. All can be competed specifically by the lysozyme sequence (−96/−66) and by two different Sp1 binding sites [one from the SV 40 promoter (Sp1 SV) and one from the dihydrofolate reductase gene (Sp1 dhfr)], except for one minor band. An AP4 binding site cannot compete. This complex pattern of several shifted bands is typical for Sp1 as can be seen in the reciprocal experiment (figure 3): the labelled Sp1 dhfr DNA shows the same pattern of shifted bands which is competable by itself, the Sp1 SV and the lys −96/−66 oligonucleotides. The Sp1 and lys −96/−66 oligonucleotides differ only in their binding affinities, that of the lysozyme sequence being weakest.

Tissue Specific Differences in Binding Patterns of Nuclear Proteins

In order to identify those regions of the lysozyme enhancers involved in binding tissue specific proteins, we compared nuclear extracts from a chicken liver cell line (DU249, in which the lysozyme gene is inactive) with the HD11 promacrophage extract. Some extract specific differences can be detected for both enhancer elements in their promoter distal parts (figure 4). The E-2.7 element manifests its specificity in HD11 specific hypersensitive sites within the large footprint area between nucleotides −2635 and −2675 (figure 4A: domain A). Cell type specific footprint patterns can be seen on the E-0.2 element between nucleotides −155 and −192 (figure 4B: domain A). One HD11 specific footprint (−155/−175) and one extended footprint caused by the DU249 extract (−180/−192) are evident (figure 4B).

The extended footprint seen in the region −193 bp to −180 bp with the DU249 extract was also seen with an extract from another lysozyme negative cell line, HD3 erythroblasts (Baniahmad, Steiner and Renkawitz, unpublished results). This footprint may be the result of a single additional binding protein absent in HD11, or may reflect the binding of a completely different set of binding proteins in each extract. To distinguish between these two possibilities, we carried out footprinting reactions with the liver cell extract in the presence of an excess of various competitor oligonucleotides (figure 5). As a control we used the oligonucleotide −208/−162 (oligonucleotide 1, see figure 2) which covers the liver cell-specific footprint plus both flanking footprints. Upon competition this complete

Figure 6. Regions of the E-2.7 kb and the E-0.2 kb enhancer bound by nuclear proteins. Binding sequences as determined by footprinting and band shift experiments are boxed. DNA regions covered by the progesterone (PR) or glucocorticoid (GR) receptors are indicated (1,22). Sequences with identical shading can compete each other. The table lists all the lysozyme sequences (underlined) protected in footprint experiments (numbered E-2.7F1 to F5 and E-0.2F1 to F6) with their similarities (capital letters) to known transcription factor binding sites (see text). AP3-C/EBP binding sequences are either taken from SV40 (26) or from polyoma (28). (+) and (−) indicate the presence or absence of a particular footprint with extracts from promacrophages (M) or from liver cells (L).

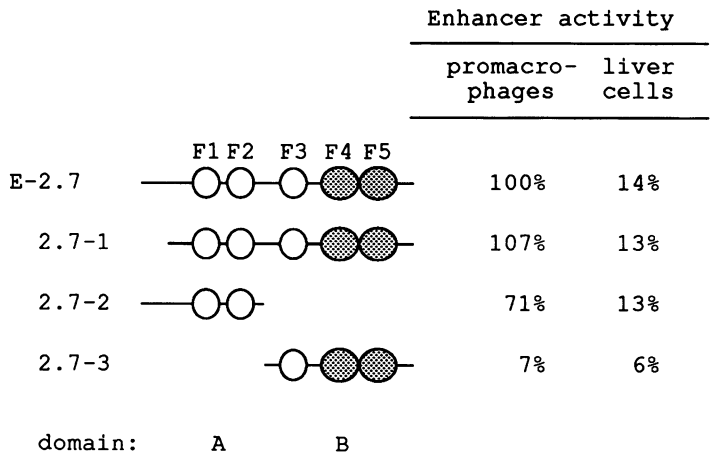


Figure 7. Functional activity of E-2.7 kb enhancer derivatives. The E-2.7 element and subregions thereof were cloned in front of tk CAT and transfected into HD11 (promacrophages) or DU249 (liver) cells. CAT expression is normalized to the expression of tk CAT and enhancer activity of E-2.7 in HD11 is set to 100%. Footprint regions F1 to F5 are shown. The function of domains A and B is discussed in the text.

region becomes DNaseI sensitive. However, competition with the short oligonucleotide −193/−180 (oligonucleotide 5, see figure 2), which covers only the liver cell specific footprint, generates a cluster of DNase I-sensitive sites in this central region without affecting the flanking footprints. This pattern of protected regions is identical to that achieved with the macrophage extract in the absence of competitor (figure 5). Therefore, we conclude that this extract-specific difference in the footprint patterns is caused by an additional factor binding on the DNA between −193 bp and −180 bp upstream of the transcriptional start site, this factor being absent in promacrophage nuclei.

Figure 6 summarizes the footprinting results described above. For each of the lysozyme sequences protected in the footprinting experiments (E-2.7F1 to F5 and E-0.2 F1 to F6) similarities to other nuclear factor binding sites are indicated. E-2.7F1 contains two repeats of the GCN4 protein binding site which is the yeast homologue to transcription factor AP1 (23). This particular binding site has only a low affinity for GCN4 (24), but since it is repeated, both may function in a synergistic manner. E-2.7F2 is quite similar to the SV40 PU-box, which can be bound by a lymphoid specific protein (25). E-2.7F3 contains a strong binding sequence for GCN4-AP1 (24). Sequences in E-2.7F4, E-0.2F4 and F5 are similar to each other (lys.cons.) (5) and to the 'core' enhancer sequence (21). This motif can be bound by the AP3 protein (26,27) or by C/EBP (28). In addition E-0.2F4 is similar to a 'half binding site' of NF1 (15). E-2.7F5 shows similarities to AP3-C/EBP and to the 'octa'-sequence (29,30). AP3-C/EBP similaritiy is also seen with the footprint sequences E-0.2F1 and F3 with F3 containing a CAAT-box, which is bound by C/EBP as well (31). All of the footprint sequences with AP3-C/EBP similarity are competeable by the SV40 'core'-sequence (see above) which is recognized by AP3 (27). The footprint E-0.2F2 contains a sequence identical to the negative NRDI region of the human β-interferon gene (32) and E-0.2F6 contains a binding sequence identical to the Sp1 binding site of the human U2 small nuclear RNA gene (33). In addition to the regions defined by the footprinting

experiments with whole nuclear extracts the glucocorticoid and progesterone receptor binding sites are shown on the E-0.2 enhancers (7,34).

Since tissue specific footprinting patterns are seen on the distal halves of each enhancers, we carried out functional tests both on the complete enhancers and on isolated enhancer elements.

Tissue Specific Enhancer Activity is Achieved by a Combinatorial Effect of Enhancer Modules

Having found multiple binding sites for similar nuclear factors on both enhancers, we wanted to know whether or not all of these sites are required for function, and if interaction between sites could be detected. We cloned several enhancer subfragments in various orientations and combinations upstream of the thymidine kinase (tk) promoter, which in turn was fused to the chloramphenicol acetyl transferase (CAT) gene (10). The resulting contructs (figures 7 and 8) were transfected into chicken liver cells (DU249) and chicken promacrophages (HD11), and cell extracts were analyzed for CAT-activity. To normalize for different transfection efficiencies into these two cell lines, we used a plasmid carrying the SV40 enhancer upstream of the tkCAT fusion gene as a standard. Both lysozyme enhancers showed about half of the SV40 enhancer activity in HD11 cells (data not shown), whereas they remained inactive in DU249 cells (figures 7 and 8).

The E-2.7 enhancer either intact, or with a 5′ deletion (2.7−1), which does not affect the footprint region, shows full enhancer activity in promacrophage cells and low activity in liver cells (figure 7). The two enhancer parts (2.7−2 and 2.7−3) show very different activities: the distal domain (A) is clearly active by itself, but the proximal domain (B) is not. Only the combination of both (2.7−1) leads to full activity in HD11 promacrophages. Similarly, dissection of the E-0.2 enhancer demonstrates the function of two domains (figure 8). 3′ deletions up to domain A (construct 0.2−2) show a progressive loss of enhancer activity indicating the requirement of domain B for enhancer function. Neither domain A nor B is active in isolation. Duplication leads to strong activity of domain A (0.2−2×2), whereas domain B (0.2−2×3) remains inactive. Interestingly, duplication of 'A' shows wildtype enhancer activity in HD11 promacrophages, but does not show the tissue specificity: clear enhancer activity is seen even in liver cells (figure 8A).The recombined enhancer with both halves arranged in a head to head orientation (construct 0.2−4) maintained wildtype enhancer activity and tissue specificity. Upon separation of the two halves by 23 bp with the distal domain in sense orientation (construct 0.2−5) or by 46 bp with the distal domain in *anti*-sense orientation (construct 0.2−6) enhancer activity was considerably reduced.

In order to confirm that the observed CAT-activities reflect the amount of correctly initiated RNA, RNase mapping experiments were carried out on RNA generated after transfecting selected constructs in HD11 cells. These experiments confirmed the results of the CAT-assays (figure 8B).

To examine in more detail the observed disruption of enhancer activity following separation of the enhancer halves, we inserted linkers of different length (5, 8, 10, 13, 15, 17 and 20 bp) between positions −161 bp and −162 bp. CAT-activities recorded after transfection into HD11 cells are shown in figure 9. Insertion of 5 bp reduced enhancer activity considerably, with a further reduction caused by an 8 bp spacer. However, a spacer of 10 bp leads to an increase in enhancer activity, presumably allowing the proteins bound to the two enhancer halves to be positioned in a three dimensional arrangement similar to that in the wildtype enhancer. This result indicates the requirement for a certain distance of the enhancer domains in order to function optimally. Further separation of the elements

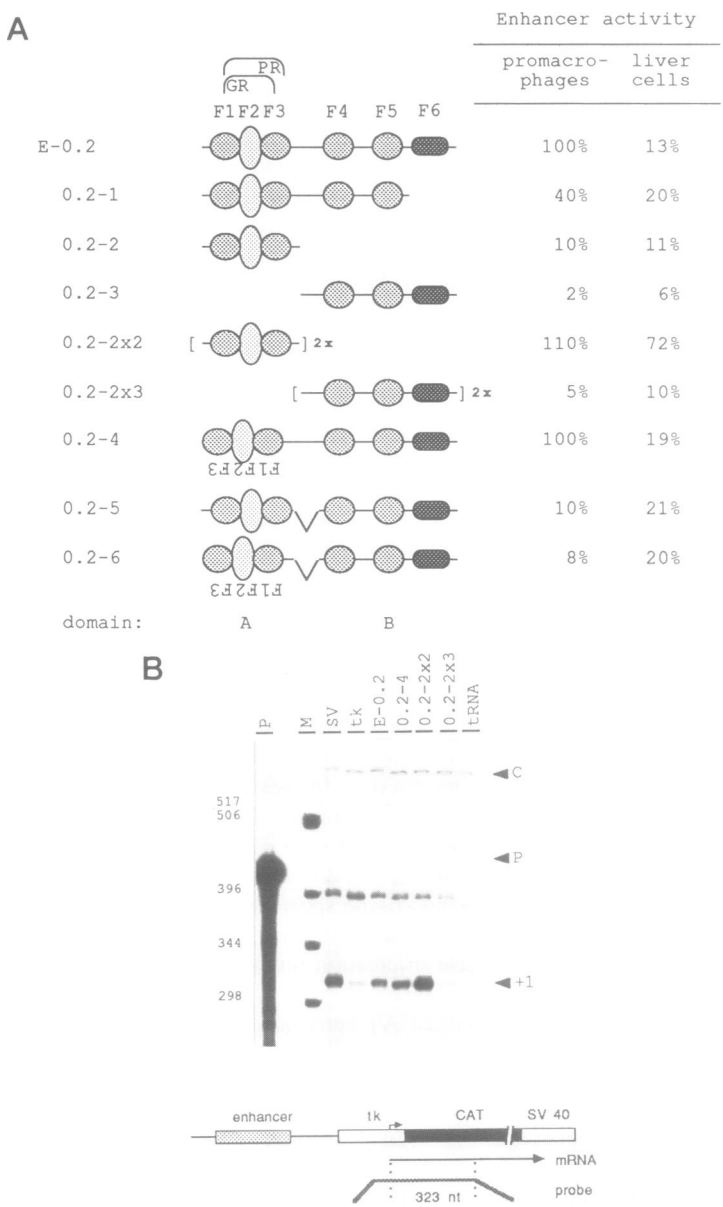


Figure 8. Functional activity of E-0.2 kb enhancer derivatives. E-0.2 recombinant sequences were cloned in front of tk CAT and transfected into HD11 or DU249 cells (see legend for figure 7). **A.** CAT activity. Footprint regions F1 to F6 and binding sites for the progesterone (PR) and glucocorticoid (GR) receptors are indicated. **B.** RNase mapping of selected constructs after transfection into HD11 cells. Lane (P) is the untreated probe, lane (M) end-labelled marker DNA and lane (tRNA) the probe hybridized with tRNA and digested with RNase A and T1. (C) is a labelled control DNA fragment carried through the RNase mapping procedure to judge complete recovery of nucleic acids. (+1) indicates the position of correctly initiated RNA and (P) the size of the probe. The diagram depicts the arrangement of expression plasmid, of the transcript and of the RNA probe.

resulted in levels which, although suggestive of a periodicity in activities, lie within the deviation of the experiment.

We conclude that activity of the 150 bp E-0.2 kb enhancer is a result of a synergistic, orientation independent interaction between domains A and B, and that tissue specificity is achieved by the combinatorial effect of both domains.

Both Enhancer are LPS Inducible

Since lysozyme expression is gradually increased during macrophage differentiation (2) and since both enhancer elements show a higher activity in primary mature macrophages as compared to HD11 promacrophages (5) we analyzed the enhancer activity in LPS treated HD11 cells. LPS is known to activate and to induce developmental changes in macrophages [for review see (35)]. To test, whether the activity of the lysozyme enhancer elements is sensitive to LPS treatment of the cells, we carried out DNA transfer experiments in the presence of LPS. Transient expression of pE-0.2 tk CAT and pE-2.7 tk CAT in HD11 cells was clearly induced by LPS, whereas the ptkCAT control plasmid was not induced (figure 10).

DISCUSSION

The complex regulation of the chicken lysozyme gene by steroid hormones in the oviduct and by the maturation of macrophages is achieved by the combined action of several enhancer, silencer and steroid responsive elements [for review see (36)]. Whether all of these elements act independently on the promoter or whether they interact with each other remains to be shown. Here we describe the detailed analysis of both lysozyme gene-enhancers E-2.7 kb and E-0.2 kb, which consist of several protein binding sites coinciding with functional modules. Some of the binding proteins are tissue-specific, others are present in liver cells as well as in promacrophages. Such a concomitant binding of cell-type specific and ubiquitous factors is also found in other enhancer elements, for example in that of the mouse α1-antitrypsin gene (37). The lysozyme enhancers E-2.7 kb and E-0.2 kb together contain six 'core' motif homologies which are completely protected by nuclear proteins from digestion with DNaseI (figure 6). Competition experiments demonstrate the importance of the 'core' homology in the binding, as cross-competition is a property of all protected regions which share only these sequences. This indicates that proteins with at least similar DNA-binding domains recognize different parts of both enhancers E-2.7 kb and E-0.2 kb. Although we find multiple similar binding sites, the enhancer domains show different activities, and specificities and therefore the binding proteins may be different. It is possible that the binding proteins described here are related to the previously characterised factors AP3 or C/EBP (26,28), although this has to be proven by analyzing the respective close contact sites on the recognition sequences. A similar puzzling situation of cell type specific and unspecific effects caused by the same binding sequence has been found for the octamer sequence [for review see (38)]. Both the expression of B-cell specific and of 'housekeeping genes' can be driven by octamer factors.

Characterization of the protein-binding sites has prompted us to dissect E-0.2 kb into two domains. Although neither of these domains has strong enhancer activity alone as a single copy, duplication of the distal domain ($-208/-162$) results in an activity similar to that of the complete enhancer, but without its tissue specificity. Only the combinatorial effect of both domains leads to wildtype activity and specificity. This was also shown for the two domains of the E-2.7 kb enhancer (figure 7). The synergistic effect of enhancer domains was further substantiated by the detection of enhancer activity even with completely

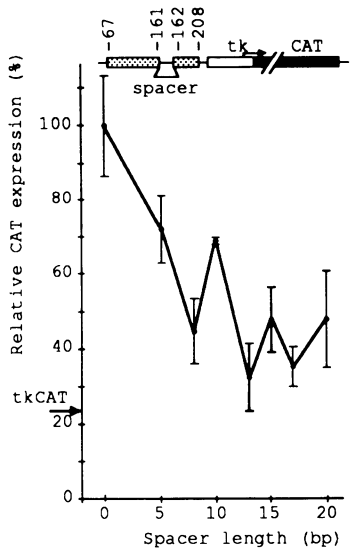


Figure 9. CAT activities of spacing mutants of the E-0.2 kb enhancer. Different spacer lengths were inserted between nucleotides −162 and −161 of the E-0.2 kb enhancer and tested for their effect on enhancer activity upstream of tk CAT. CAT activities achieved after transfecting HD11 cells in triplicate experiments are shown with standard deviations, activity of tk CAT without enhancer sequences is indicated by the arrow.

rearranged modules. Investigation of the requirement for a particular distance of the two domains indicated a dependence of enhancer activity on the relative position of the modules. Similar effects of the stereospecific alignment on the efficiency of transcription regulating elements has been shown for the SV 40 enhancer (39) or for the interaction of the glucocorticoid receptor and the CACCC-protein (14). In each case optimal activity was only observed after introduction of multiples of a complete helical turn of the DNA between the protein binding sites. In the case of lys E-0.2 kb, we observe a strong decrease of activity upon introducing additional nucleotides between its two domains with a peak of increased activity upon insertion of 10 bp. A synergistic interaction of the bound proteins in this constellation would be indicative of a bending of DNA between the two domains, a flexible protein domain and/or the involvement of a 'bridging protein' that mediates the interaction of the DNA-bound factors and could confer the flexibility to the whole system. Participation of non-DNA-binding proteins in transcription regulation has been shown in some cases (40−44).

Domain structures such as those described here are also found in other transcription regulatory elements and seem to be a general phenomenon (37,45,46). A modular structure may be adapted during evolution to a variety of regulatory processes by using various combinations of a limited number of different binding sites.

Clear tissue-specific differences in the binding pattern of liver cell and promacrophage extracts were observed between −2675 bp and −2635 bp and −192 bp and −155 bp (figure 6). The regions of both enhancers (domain A) harboring these binding sites show enhancing activity by themselves or upon duplication in promacrophage cells (figures 7

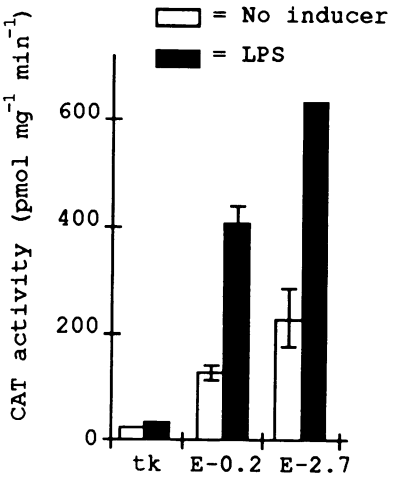


Figure 10. LPS induces enhancer activity. Both enhancer constructs pE-0.2 tk CAT (E-0.2) and pE-2.7 tk CAT (E-2.7) were transfected into LPS treated or untreated HD11 promacrophages. CAT activities are compared to the expression at ptk CAT (tk).

and 8). Whether the increase of enhancer activity during differentiation or during LPS treatment involves one or several of these binding proteins remains to be shown. In the case of E-0.2 kb it seems that liver cells contain an extra DNA binding protein in addition to those found in the promacrophage line. A possible negative effect of this protein is also suggested by the remarkable similarity of the binding sequence with the negative regulatory domain I (NRDI) of the human β-interaction gene (32).

The E-0.2 kb enhancer of the chicken lysozyme gene can also bind steroid-hormone-receptors in vitro, and is involved in the hormonal control of lysozyme synthesis in the oviduct [(7) and figure 6]. Since the receptor binding sites are overlapping with the nuclear factor binding sites described in this paper, a degree of interference between regulatory proteins may be postulated, possibly involving a block of expression in the oviduct being overcome by a steroid stimulus. In other cells, although the appropriate receptors are present, nuclear factors might prevent binding of steroid receptors. Such a tissue specific steroid regulation has recently been demonstrated for the chicken ovalbumin gene (47). The E-0.2 kb enhancer evidently acts as a multifunctional element: it contributes to the constitutive gene expression in macrophages and the hormone-induction in the oviduct, the regions mediating these effects being structurally, and probably functionally, highly integrated.

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