

The developmental activation of the chicken lysozyme locus in transgenic mice requires the interaction of a subset of enhancer elements with the promoter

Matthias C. Huber, Ulrike Jäggle⁺, Gudrun Krüger and Constanze Bonifer*

Institut für Biologie III der Universität Freiburg, Schänzlestraße 1, D-79104 Freiburg, Germany

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ABSTRACT

The complete chicken lysozyme locus is expressed in a position independent fashion in macrophages of transgenic mice and forms the identical chromatin structure as observed with the endogenous gene in chicken cells. Individual lysozyme *cis*-regulatory elements reorganize their chromatin structure at different developmental stages. Accordingly, their activities are developmentally regulated, indicating a differential role of these elements in locus activation. We have shown previously that a subset of enhancer elements and the promoter are sufficient to activate transcription of the chicken lysozyme gene at the correct developmental stage. Here, we analyzed to which grade the developmentally controlled chromatin reorganizing capacity of *cis*-regulatory elements in the 5'-region of the chicken lysozyme locus is dependent on promoter elements, and we examined whether the lysozyme locus carries a dominant chromatin reorganizing element. To this end we generated transgenic mouse lines carrying constructs with a deletion of the lysozyme promoter. Expression of the transgene in macrophages is abolished, however, the chromatin reorganizing ability of the *cis*-regulatory elements is differentially impaired. Some *cis*-elements require the interaction with the promoter to stabilize transcription factor complexes detectable as DNase I hypersensitive sites in chromatin, whereas other elements reorganize their chromatin structure autonomously.

INTRODUCTION

Different types of regulatory mechanisms contribute to the tissue- and development-specific regulation of a gene. It has long been known that upstream *cis*-regulatory elements binding a variety of *trans*-acting factors and promoters directing assembly of the basal transcription machinery, are essential for correct gene activation. In recent years, it has emerged that the chromatin structure of eukaryotic genes may present an efficient additional regulatory layer of gene expression. Nucleosomes in regulatory regions of eukaryotic genes are often precisely placed at crucial

positions of *cis*-regulatory elements (1,2). This may generate DNA conformations accessible only for certain transcription factors, since some of them are unable to bind to recognition sequences which are organized within a nucleosomal core (3–6). It was further suggested that by binding on a nucleosomal surface, regulatory proteins are aligned into defined orientations (3,4,6–11). Promoters as well as enhancers can be organized in specific nucleosomal conformations (12–16). The mouse serum albumin enhancer is organized in an array of three positioned nucleosomes, however, only in liver chromatin, where the enhancer is active and bound by transcription factors. Here, nucleosome positioning is determined by DNA binding factors which stabilize one of three translational positions (14,17). Chromatin remodeling during gene locus activation seems to be influenced by several different mechanisms. For example, the acetylation of histone N-termini facilitates transcription factor binding (18,19). Furthermore, enzymatic activities have been identified that assist transcription factors to reconfigure chromatin in an ATP-dependent manner. These include the SWI/SNF complex of yeast and related complexes of higher organisms (20–24).

How are such chromatin reconstruction processes initiated, and by which *cis*-regulatory elements are they controlled? Earlier investigations of promoter mutants in the yeast HSP82 gene demonstrated an uncoupling of transcription from DNase I hypersensitive site (DHS) formation, since a DHS is formed at a promoter, which is inactivated by a mutation (25). In contrast, experiments analyzing constructs of the chicken β -globin gene in transgenic mice demonstrated that DHS formation at the 3'-enhancer/locus control region (LCR) was shown to be dependent on the presence of a promoter within or adjacent to the transgene (26). Recent studies aimed at clarifying the role of enhancers in chromatin present evidence for a probability model of enhancer activation (27–29). In this model, enhancers increase the probability of forming a stable transcription complex at the promoter by antagonizing repressive chromatin structures. Experiments supporting this idea showed that the formation of a DHS at an enhancer element is an all-or-none mechanism (30). Similar rules guide chromatin reconfiguration in more complex systems. The LCR is essential for the formation of active chromatin in the human β -globin locus (31–34). It could be shown that the LCR, which is composed of several individual DHS, is able to switch its interaction between different promoters. The stability of

*To whom correspondence should be addressed at present address (after 1 September 1997): Molecular Medicine Unit, University of Leeds, St James's University Hospital, Leeds LS9 7TF, UK. Tel: +44 113 206 5681; Fax: +44 113 244 4475

⁺Present address: Erasmus University Rotterdam, Faculty of Medicine, Department of Cell Biology and Genetics, PO Box 1738, 3000 DR Rotterdam, The Netherlands

LCR–promoter interaction is dependent on the completeness of the LCR (35,36). However, the molecular details of LCR–promoter interaction have not yet been elucidated.

The lysozyme gene is specifically expressed in the myeloid lineage of the hematopoietic system and is regulated by a combination of several *cis*-regulatory elements, all located in the 5'-half of the locus. The complete chicken lysozyme locus carrying the full set of regulatory elements is expressed at a high level and independent of the genomic integration site in transgenic mice (37). Three enhancers, 6.1, 3.9 and 2.7 kb upstream of the transcriptional start site, as well as a negative regulatory (silencer) element at -2.4 kb and a complex promoter (38–45), have been identified. All active *cis*-regulatory elements colocalize with DHSs in chromatin (46–50). Deletion of one enhancer region abolishes position independence of expression (51). Repression of gene expression by genomic position effects is correlated with suppression of DHS formation and leads to an inefficient reorganization of nucleosomes in the *cis*-regulatory regions (15,52), indicating that active chromatin formation and transcriptional activity are closely linked. According to their developmental stage of activation, the individual enhancer elements of the lysozyme locus can be categorized into early or late enhancers. The early enhancers at -6.1 and -3.9 kb and the promoter become DNase I hypersensitive at the myeloblast stage when the gene is transcriptionally activated. The DHS at the silencer element is still present. The DHS at the late -2.7 kb enhancer appears only later in differentiation, at the promacrophage stage, simultaneously, the -2.4 kb silencer disappears (48,49). Together with the promoter, each enhancer is capable of activating the gene locus specifically in mature macrophages (51,53). However, the temporal regulation of their activity is different, since the early enhancers and the promoter are sufficient to activate the chicken lysozyme gene at the correct, early developmental stage, whereas a deletion of the early -6.1 kb enhancer leads to a delay in gene activation (53). This indicates that the early enhancers are responsible for the activation of the lysozyme locus in early macrophage precursor cells.

To understand the contribution of individual *cis*-regulatory elements to locus activation, it is important to elucidate their mutual dependencies with regard to their chromatin reconfiguration capacities. In particular, we wanted to know whether chromatin in the 5'-regulatory region of the lysozyme locus can be reorganized in the absence of a promoter. To this end, we generated transgenic mouse lines carrying a chicken lysozyme gene domain without the promoter region. While deletion of promoter sequences completely abolishes expression of the transgene, DHS formation at the different *cis*-regulatory regions is differentially impaired. The early enhancers require the interaction with the promoter for chromatin reconfiguration, whereas the late enhancer and the silencer do not.

MATERIAL AND METHODS

Construction of pIIIlys P-

The promoter-less lysozyme locus (pIIIlys P-) was constructed by cloning a PCR generated fragment, covering the region between +1 and +250 bp, blunt into the *EcoRV* site of vector poly IIIi (54) (construct pIIIiUTR). The 5'-PCR primer contained an extension with a *SalI* site. In a second cloning step a 5.6 kb *SacI* fragment (S3–S4) from pIIIlys (37) was inserted into the juxtaposed *SacI* of pIIIiUTR and named pIIIiP-S3S4. In a third

cloning step an *SphI*–*XbaI* fragment (Sp2–X4) from poly IIIiUTR was cloned into pIIIiUTR cleaved with *SphI* and *XbaI* (55). The resulting construct was cleaved with *SalI*, and the *SalI* fragment covering the 3'-half of the lysozyme locus was cloned into pIIIiP-S3S4 cleaved with *SalI*. The resulting construct as well as pIIIiUTR were cleaved with *SpeI*. The *SpeI* fragment in pIIIiUTR covering the promoter was exchanged against the *SpeI* fragment originating from the promoter-less construct. To generate a unique *SalI* site at +1, the 3'-*SalI* site from the pIIIi polylinker was mutated.

Transgenic mice and cell culture

Production of the P- transgenic mouse lines by pronuclear injection of DNA was essentially performed as described in (56). First-generation heterozygous mice from the founders P-0, P-1, P-4 were examined for intact integration and construct integrity by Southern blotting. Copy-numbers were calculated from Southern blots as described in (37) with the help of a phosphor-imager. Expression and chromatin analysis were performed with homozygous progeny. Transgenic mouse lines carrying construct XS (51) were kept as homozygous lines in our own mouse colony. Primary macrophages were prepared from the peritoneal cavity of transgenic mice and were stimulated with bacterial lipopolysaccharide (LPS) as described (37). For each transgenic mouse line cells from 12–20 mice were taken in culture in standard Iscove's medium supplemented with 10% fetal calf serum (FCS) and 10% L-cell conditioned medium for 16 h (37). Embryonic fibroblasts were prepared from mouse embryos 12 days after fertilization as described earlier (52). HD11 cells were grown in standard Iscove's medium containing 8% FCS and 2% chicken serum.

mRNA expression analysis

Preparation of mRNA and the S1 protection analyses were performed as described in (37). For RT-PCR analysis primary peritoneal macrophages were cultured as described above for 1 day. Where indicated, the samples were treated by adding bacterial lipopolysaccharide (LPS/Sigma) at 5 µg/ml. Total RNA was isolated using 0.5 ml RNAzol™ B (Biotecx Laboratories, Inc.) according to the manufacturer's instructions. cDNA of isolated total RNAs from the different samples was prepared using random hexamers as primers and Moloney Murine Leukemia Virus Reverse Transcriptase (Gibco-BRL) in a reaction volume of 20 µl under conditions recommended by the manufacturer. Two units of Ribonuclease Inhibitor (Gibco-BRL) were added per reaction. cDNA was subsequently heated to 70°C for 5 min to inactivate reverse transcriptase. To ensure the use of comparable amounts of RNA and cDNA for the different samples, the relative expression level of the HPRT gene was used as a standard for calibration. After measurement of HPRT expression 1:10 dilutions of the corresponding samples were used to determine the expression level of mouse lysozyme and the transgene. Primers used were: HPRT: 5'-CACAGGACTAGAA-CACGTGC-3'; 5'-GCTGGTGAAAAGGACCTCT-3'; mouse-lysozyme (m-lys): 5'-ACCCAGCCTCCAGTCACCAT-3', 5'-CAGTGCTTTGGTCTCCACGG-3'; chicken-lysozyme (c-lys): 5'-GATCGTCAGCGATGGAAACGGC-3', 5'-CTCACAGCCG-GCAGCCTCTGAT-3'. HPRT-PCRs were performed with 1.25 mM MgCl₂ and 18 pmol of each primer. PCRs for mouse-lysozyme were performed with 1.25 mM MgCl₂ and 15 pmol of each

primer, whereas chicken lysozyme PCRs were performed with 1.0 mM MgCl₂ and 15 pmol of each primer. To every PCR reaction, 1 µl of the corresponding cDNA dilution was added. PCR reactions were done in a total volume of 30 µl and with 1.5 U *Taq* polymerase (Gibco-BRL). PCRs were carried out in a Trio-Thermoblock (Biometra) using a regimen of 94°C for 40 s, 55°C (HPRT) or 62°C (m-lys and c-lys) for 40 s and 72°C for 1 min for 35 cycles. Samples were loaded onto a 6% PAG. Gels were stained with ethidiumbromide and photographed under 245 nm UV light.

Nuclei preparation

Nuclei were prepared by homogenizing cultured cells on ice with a Dounce homogenizer in buffer 1 (0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris-HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 500 mM Sucrose, 1 mM PMSF) followed by centrifugation for 5 min at 1000 g at 4°C. Nuclei were washed once in buffer 2 (buffer 1 + 0.5% Triton X-100), followed by a wash in buffer 3 (buffer 1 but with 350 mM sucrose instead of 500 mM). After this wash nuclei were centrifuged for 5 min at 600 g at 4°C.

DNase I and MNase digestion analysis

Aliquots of 2×10^7 to 1×10^8 nuclei in 100–200 µl of buffer 3 were centrifuged for 5 min at 600 g and 4°C and thereafter resuspended in buffer 4 (0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris-HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA). DNase I digestions were performed in 500 µl buffer 4. To 2×10^7 nuclei 0, 4, 10, 20 and 40 U/ml DNase I (Pharmacia) were added. HD11 nuclei were digested with 24 U/ml DNase I. Digestion was started by adding 4 mM MgCl₂ and 2 mM CaCl₂. Incubations (15 min, 4°C) were stopped by adding 10 µl 0.5 M EDTA. MNase digestions were performed in 200 µl buffer 4. To 2×10^7 nuclei 0, 15, 80 U MNase (Pharmacia) were added. Digestion was started by adding 10 µl CaCl₂ (100 mM) and stopped after incubation (5 min, 25°C) by the addition of 10 µl 0.5 M EDTA. Digestion of naked genomic DNA with MNase was performed in 150 µl 10 mM Tris-HCl pH 7.5 with 0.2–6.4 U/ml MNase. Incubations (15 min, 25°C) were started by adding 15 µl CaCl₂ (10 mM) and stopped with 15 µl 50 mM EDTA. After DNase I or MNase digestion, nuclei were lysed in 500 µl Tris-HCl pH 8.0, 2 mM EDTA, 0.2% SDS, 0.5 mg/ml Proteinase K and incubated overnight at 37°C. RNase A (0.2 mg/ml) was then added and after a further incubation at 37°C for 1 h the DNA was precipitated three times with ethanol. Digested DNA was cleaved with restriction enzymes for indirect endlabelling analysis and 7–30 µg of fragmented DNA were loaded on 3 mm thick vertical 1% agarose gels (DNase I analysis) or 10 mm thick vertical 1.5% agarose gels (MNase analysis). The DNA was transferred to Biodyne B membrane and the filter was hybridized with an appropriate probe for indirect endlabelling. For DHS-analysis, probe A (1.65 kb *Hind*III fragment) and probe B (0.29 kb *Hind*III fragment) were used. For MNase analysis, probe 1 (*Sph*I–*Spe*I fragment from –3163 to –2906 bp), probe 2 (*Dra*II–*Sph*I fragment from –3424 to –3163 bp) and probe 3 (*Sac*I–*Bam*HI fragment from –6492 to –6331 bp) were used.

RESULTS

The generation of transgenic mouse lines carrying a chicken lysozyme locus without a promoter

To examine the consequences of the removal of the chicken lysozyme promoter on gene expression and the chromatin structure of the 5'-regulatory region, we generated three transgenic mouse lines (P-0, P-1, P-4) carrying different copy numbers of a chicken lysozyme locus in which sequences between –830 and +1 had been deleted (Fig. 1A). The deletion removes all three TATA and C/CAAT boxes (57) and all upstream macrophage-specific factor binding sites (58,59) as well as sequences covering the DHS at –0.7 kb. The *cis*-regulatory function of this DHS is up to now unknown. Mouse line XS.0b, carrying the XS construct containing all *cis*-regulatory elements which is expressed at a high level and in an integration-site independent manner in macrophages (51) was analyzed for comparison. The *cis*-regulatory elements of the lysozyme locus are successively activated in macrophage differentiation as schematically depicted in Figure 1A (15,49). We analyzed transgene expression and chromatin structure of mature peritoneal macrophages, representing the active expression status of the transgene in the mouse, as well as in embryonic fibroblast cells representing a lysozyme non-expressing cell type (Fig. 1B) (15).

Deletion of lysozyme promoter sequences abolishes transgene expression

In a different study examining the influence of promoter deletion on the chromatin opening capacity of the chicken β-globin 3'-enhancer/LCR (26), two of six transgenic mouse lines were found to express transgene encoded RNA, presumably starting from an adjacent mouse promoter. Such 'read through' transcription obviously influenced the chromatin structure of the remaining enhancer/LCR region and led to strong DNase I hypersensitivity. In order to exclude such an artificial expression which would influence chromatin reconfiguration at the *cis*-regulatory elements, we examined numerous tissues of P- mice for the presence of lysozyme mRNA by S1-protection analysis (Fig. 2A, lanes 2–25). S1-analysis detected no lysozyme mRNA in any of the analyzed tissues of the investigated mouse lines. To exclude weak or antisense transcription initiated from a nearby promoter, we used the highly sensitive RT-PCR method to measure chicken lysozyme mRNA levels in macrophages of P- mice. Maximal transcriptional activation of the intact chicken lysozyme gene is achieved by treatment of macrophages with LPS, whereby all *cis*-regulatory elements except the –3.9 kb enhancer are LPS-responsive (51,58,60). We therefore measured expression levels in LPS-stimulated (Fig. 2B; lanes 5, 7 and 9) and non-stimulated (Fig. 2B; lanes 4, 6 and 8) macrophages of all P- mouse lines in comparison to that of the XS.0b mouse line (Fig. 2B; lanes 2 and 3). As controls, the expression levels of the endogenous mouse-lysozyme gene and the HPRT gene were measured. In contrast to the strong signal detected in XS.0b macrophages, in P- mice no transgene RNA was detectable, neither in LPS stimulated nor in non-stimulated macrophages. We therefore conclude that our promoter deletion completely abolished mRNA synthesis and, secondly, that no read-through transcription is detectable from juxtaposed promoters.

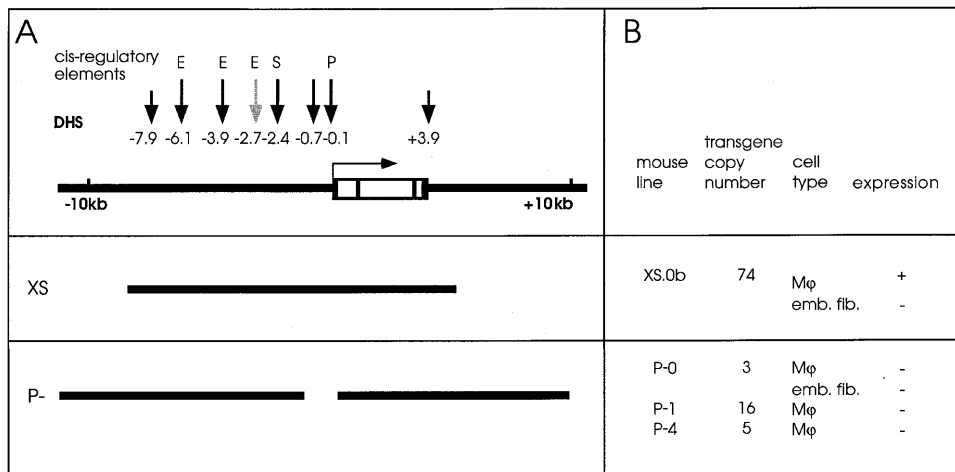


Figure 1. Transgenic mouse lines carrying a chicken lysozyme locus construct lacking the promoter region. (A) Upper panel: map of the complete chicken lysozyme locus with the coding region (large white box), *cis*-regulatory elements (at the top), DNase I hypersensitive sites (DHSs, vertical arrows). The short vertical arrows present constitutive DHSs, the black vertical arrow indicates the DHS at the silencer element (only absent in mature macrophages), the grey arrows display the DHSs that appear at the myeloblast stage and the light grey arrow the -2.7 kb enhancer DHS that appears late (in mature macrophages). E, enhancer element; S, silencer element; P, promoter elements. Middle and lower panel: size of the two different constructs (XS, P-) analyzed in transgenic mice. The gap in the P- construct indicates the promoter-deletion spanning from $+1$ to -830 bp. (B) Names of the different mouse lines, copy numbers, cell types investigated and qualitative expression status. Mφ, macrophage; emb.fib., embryonic fibroblast.

The -2.4 kb silencer and the -2.7 kb enhancer form DHSs and rearrange chromatin independently of the presence of promoter elements

In macrophages of mouse line XS.0b in which the chicken lysozyme transgene is expressed at a high level, DHSs at the promoter, the -2.4 kb silencer, the -2.7 kb enhancer, the -3.9 kb enhancer and the -6.1 kb enhancer are detectable (Fig. 3A and B, lane 7, respectively; Fig. 4B, lane 21), whereas in lysozyme non-expressing embryonic fibroblasts only the DHS at the -2.4 kb silencer element is visible (52). We analyzed the capacity of the different *cis*-regulatory elements (with the exception of the -3.9 kb enhancer) to form a DHS and thus stably bind transcription factor complexes in macrophages of the three different P- mouse lines (Fig. 3A and B). The formation of a DHS at the -2.4 kb silencer is unaffected, an observation which confirms preliminary experiments performed in stably transfected cell culture cells (61) (Fig. 3A). We also observed the formation of a DHS at the -2.7 kb enhancer. However, its relative signal-intensity as compared to the -2.4 kb DHS is weaker than in the promoter-containing gene digested to a similar extent (Fig. 3A, lanes 6 and 7). In LPS-stimulated macrophages the DHS at the -2.4 kb silencer element disappears whereas the DHS at the -2.7 kb enhancer becomes stronger (49,60). This is not observed in P- macrophages, a comparison of the relative signal intensities of the -2.4 and -2.7 kb DHSs revealed no significant differences between LPS-treated and untreated cells (Fig. 3A, lanes 1–5 and 8–12).

In order to get further insights into the chromatin structure of the -2.4 kb silencer/ -2.7 kb enhancer region at a resolution higher than that of the DHS mapping experiments, we examined this area by MNase digestion analysis (Fig. 4). Using the same method (15) we could previously show that the -2.4 kb silencer/ -2.7 kb enhancer region is covered by several specifically positioned nucleosomes, which are indicated by a pattern of regularly spaced chromatin specific MNase cuts as indicated in Figure 4A. In embryonic fibroblasts of P-0 transgenic mice (Fig. 4A,

lanes 15–17) the MNase pattern is identical to that observed in lysozyme non-expressing cells (15); however, in concordance to the DHS mapping in macrophages of all P- mouse lines, chromatin structure is rearranged towards that of lysozyme expressing macrophages (Fig. 4A, lanes 6–14). Transcriptional activation leads to an increasing accessibility of MNase cleavage sites at -2685 , -2765 and -2800 bp, which indicates the formation of an active enhancer as displayed by XS.0b macrophages, where the gene is highly expressed (Fig. 4A, lanes 18–20). Simultaneously, a decreasing accessibility of the cleavage sites at -2480 and -2830 bp, indicative for the inactivation of the silencer element, is observed. However, in P- mouse macrophages these alterations of MNase cleavage site accessibility are less prominent than in XS.0b macrophages, demonstrating that chromatin reorganization does not occur on all gene copies. We therefore observe that MNase cleavage patterns, indicative for transgenes exhibiting either the chromatin structure of expressing cells or that of non-expressing cells, are superimposed on each other. Such mixed cleavage patterns are due to an impediment of nucleosome reorganization as a result of genomic position effects, as we have demonstrated earlier (15).

The early enhancers at -6.1 kb and -3.9 kb are unable to rearrange their chromatin conformation in the absence of the promoter

The examination of the early enhancer regions at -6.1 and -3.9 kb led to a completely dissimilar result. We reprobated the same filter used in Figure 3A, however, we were unable to detect DNase I hypersensitivity at the -6.1 kb enhancer (Fig. 3B, lanes 1–5, 8–12 and 13–17), in contrast to the situation observed in transgene macrophages with an intact chicken lysozyme gene construct (Fig. 3B, lane 7; Fig. 4B, lane 21) (52). MNase-analysis of the -6.1 kb enhancer region in lysozyme non-expressing cells of P- mice shows a series of chromatin-specific MNase cuts in the region between -5945 and -6130 bp which were indistinguishable

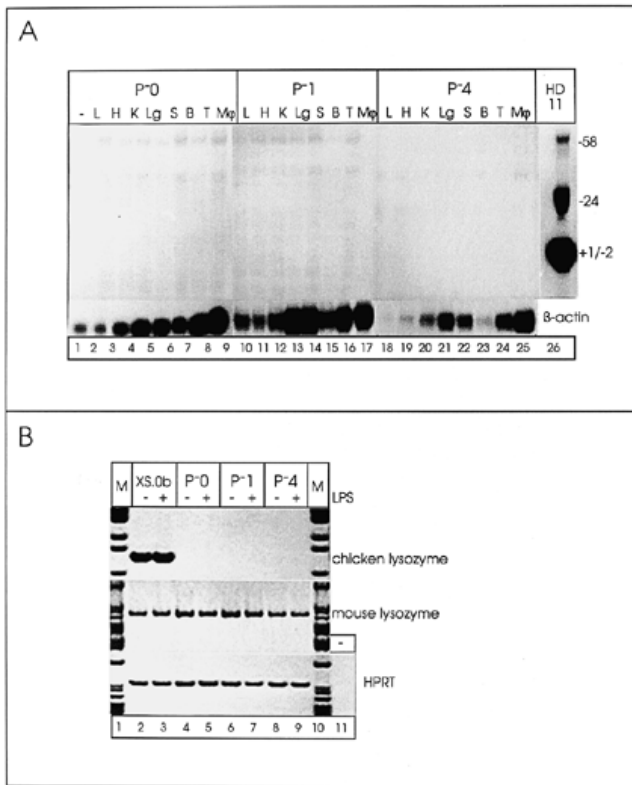


Figure 2. mRNA expression analysis of P- mouse lines. (A) Expression of the chicken lysozyme gene in different tissues of three P- transgenic mouse lines. Total RNA (20 µg) was analyzed in an S1 protection assay with probes specific for chicken lysozyme (upper panel) or mouse β-actin (lower panel) as described in Materials and Methods. Abbreviations above lanes 2–25 indicate the investigated tissues/cell types. L, liver; H, heart; K, kidney; Lg, lung; S, spleen; B, bone marrow; T, thymus; Mφ, peritoneal macrophage. Lane 1 (-), no RNA; lane 26, HD11 cells stimulated with LPS. The numbers at the right indicate the positions of the three major start sites at the lysozyme promoter (57). (B) Expression of the chicken lysozyme transgene, the endogenous mouse lysozyme and the HPRT gene measured by RT-PCR. Lanes 2 and 3, XS.0b macrophages; lanes 4 and 5, P-0 macrophages; lanes 6 and 7, P-1 macrophages; lanes 8 and 9, P-4 macrophages; lanes 1 and 10, size markers (M); lane 11, no cDNA. Cells were stimulated for 12 h with LPS where indicated. DNase I treated or untreated RNA revealed identical results. Note that RT-PCR signals with XS.0b macrophages are generated by cDNA concentrations far above the ones required to be in the linear range of the PCR reaction, since only marginal LPS induction is visible. PCR with more diluted cDNA samples demonstrated significant LPS inducibility (data not shown). The fragments sizes amplified are: chicken lysozyme 101 bp (unprocessed RNA would be 180 bp); mouse lysozyme 228 bp; HPRT 249 bp.

from the ones observed in embryonic fibroblasts of XS.0b mice (schematically indicated in Fig. 4C, left panel) (15). This pattern is rearranged in XS.0b macrophages (Fig. 4C, lanes 18–20), the most prominent differences being the disappearance of a band at -5945 bp and an increase in intensity of a band at -6130 bp. The -3.9 kb region has not yet been extensively characterized, but contains a strong enhancer element (G.Krüger and C.Bonifer, unpublished observation). In P- macrophages no chromatin reconfiguration at the -3.9 kb enhancer towards an activated chromatin status is detectable (Fig. 4B, lanes 6–14). The MNase pattern is indistinguishable from the one found in embryonic fibroblasts. The MNase pattern detected in XS.0b macrophages

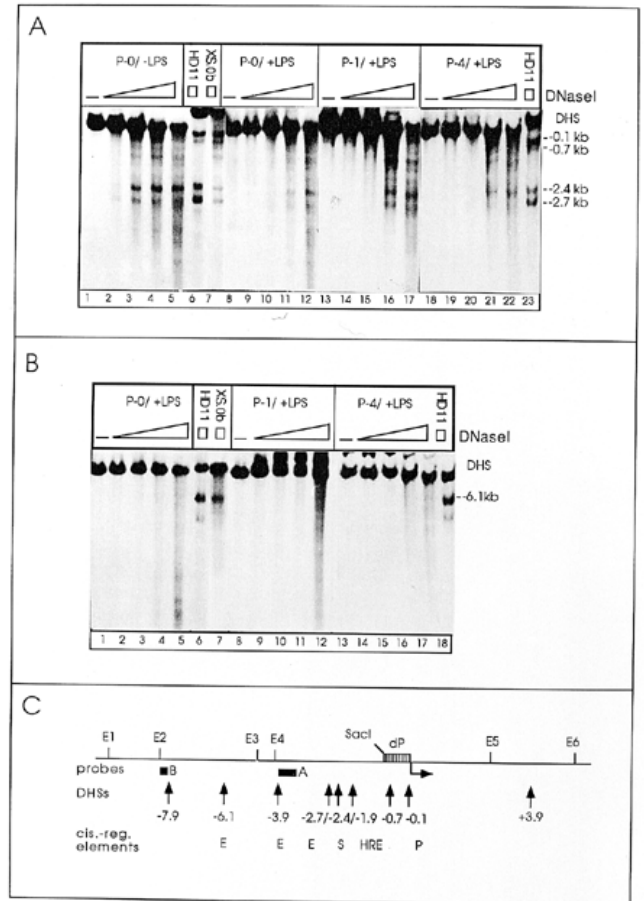


Figure 3. P- mouse lines display DHS at the -2.7 kb enhancer but not at the -6.1 kb enhancer. (A) Nuclei were prepared from untreated P-0 macrophages (lanes 1–5; 30 µg DNA/slot) and P-0 macrophages (lanes 8–12; 30 µg DNA/slot), P-1 macrophages (lanes 13–17; 7 µg DNA/slot) and P-4 macrophages (lanes 18–22; 14 µg DNA/slot), stimulated with LPS, respectively. Genomic DNA was digested with increasing amounts of DNase I, restricted with *EcoRI* and analyzed by indirect endlabelling. As reference we used DNA prepared from chicken HD11 nuclei which was digested to a similar extent (24 U/ml DNase I) and restricted with *EcoRI* (lanes 6 and 23; 30 µg DNA/slot) and XS.0b mouse macrophage nuclei which carry the intact transgene, digested with 5 U/ml DNase I and further restricted with *EcoRI* (lane 7; 10 µg DNA/slot). At the right the positions of the DHSs are displayed. The filter was hybridized with probe A. (B) The filters used in (A) were reprobbed with probe B. Lanes 6 and 18, HD11 cells; lane 7, XS.0b macrophages. At the right the positions of the DHSs are displayed. (C) Map of the lysozyme gene construct with relevant restriction sites, positions of all DHSs in the chicken lysozyme gene and cis-regulatory elements. Striped box, promoter deletion from -830 bp *SacI* restriction site to +1 bp; E1–E6, *EcoRI* sites in the lysozyme construct; black boxes (A, B), probes used in the DHS analysis relative to the *EcoRI* restriction sites; E, enhancer element; S, silencer element; HRE, hormone responsive element; P, promoter elements.

is clearly different from these, here a strong reduction in intensity of a band at -3920 bp as well as a increasing accessibility of MNase cleavage sites at -3810 and -3740 bp is observed, as compared to lysozyme non-expressing cells (Fig. 4B, lanes 19 and 20 and 15–17) (15). Taken together, our experiments demonstrate that in the absence of a promoter the early enhancers do not rearrange chromatin, although the cells contain the complete transcription factor equipment that normally suffices for chicken lysozyme gene expression.

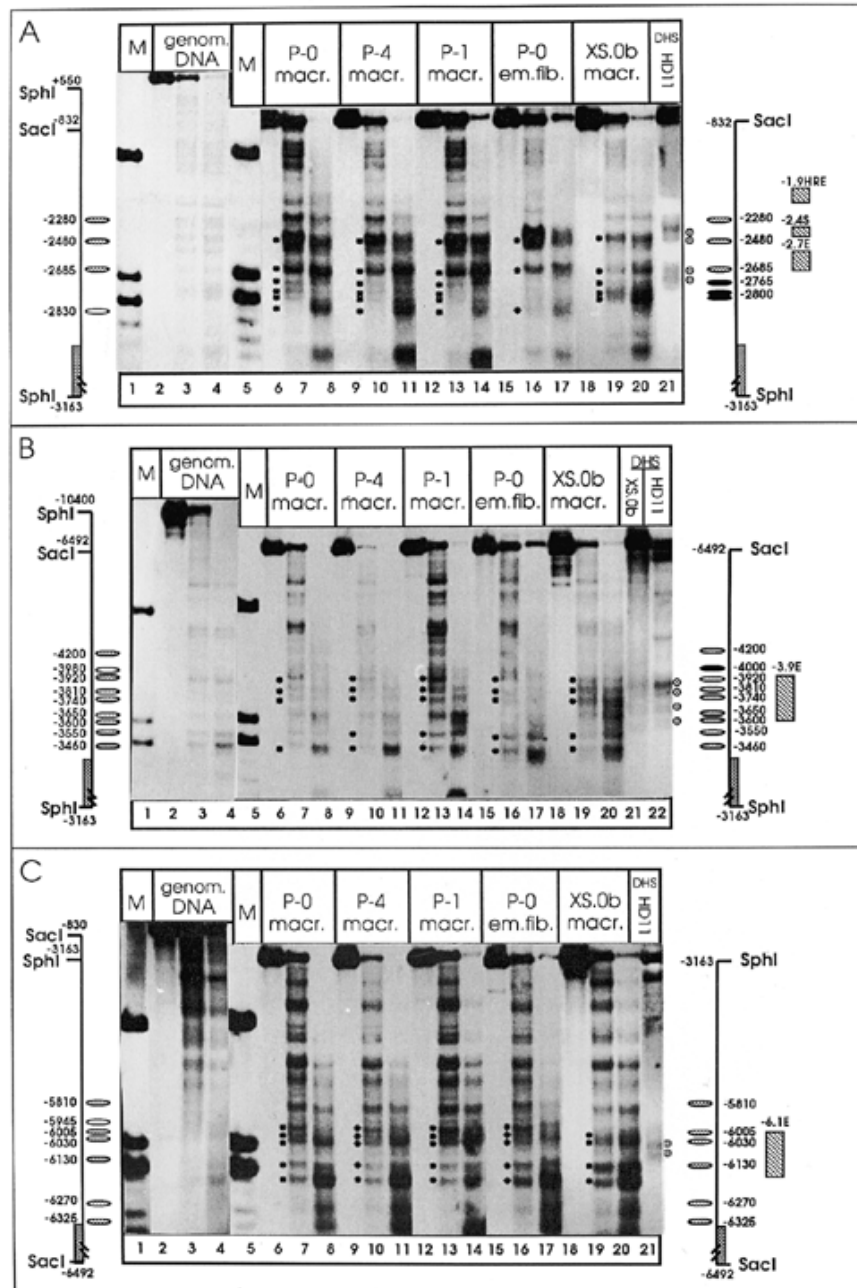


Figure 4. MNase analysis of *cis*-regulatory regions in P- mouse lines. **(A)** MNase analysis of the -2.4 kb silencer/-2.7 kb enhancer region. Lanes 2-4, MNase digestion pattern of naked genomic DNA (restricted with *SphI*); lanes 6-20, analysis of MNase digestion pattern in the chromatin of transgenic mouse macrophages (macr.) and embryonic fibroblasts (em.fib.). Genomic DNA isolated from MNase- (and DNase I-) digested nuclei was restricted with *SphI* and *SacI*. Lane 21, DHS pattern of HD11 nuclei in the analyzed region (symbolized by small grey circles). The chromatin-specific MNase digestion pattern observed in lysozyme non-expressing cells of transgenic mice carrying the intact transgene is indicated at the left, that of lysozyme expressing cells on the right. The *cis*-regulatory elements located in the analyzed region are indicated as striped boxes. Asterisks between lanes mark the MNase cleavage sites with the most significant changes (presence or absence or differences in the intensity) between transgenic mouse macrophages carrying the intact lysozyme gene (XS.0b) and P- transgenic mouse macrophages. The positions of specific MNase cuts not present in naked genomic DNA are indicated on the map (oval circles). Black oval circles mark MNase cleavage sites specific for cells expressing the transgene. White oval circles indicate MNase cleavage sites that were detected only in lysozyme non-expressing cells of transgenic mice. Grey oval circles mark specific MNase cleavage sites detected in both lysozyme expressing and non-expressing cells of mice with the intact transgene. Cleavage site positions indicated on the map are mean values of at least four independent experiments. Probe 1 is used for indirect endlabelling, its position is indicated by a stippled box. **(B)** MNase analysis of the -3.9 kb enhancer region. Analysis, description and legends are identical to those in (A) except that probe 2 was used for indirect endlabelling, its position is indicated by a stippled box. Lane 21, DHS pattern of XS.0b macrophage nuclei in the analyzed region; lane 22, DHS pattern of HD11 nuclei. **(C)** MNase analysis of the -6.1 kb enhancer region. Analysis, description and legends are identical to those in (A) except that probe 3 was used for indirect endlabelling, its position is indicated by a stippled box. M, size markers; E, enhancer element; S, silencer element; HRE, hormone responsive element; P, promoter elements.

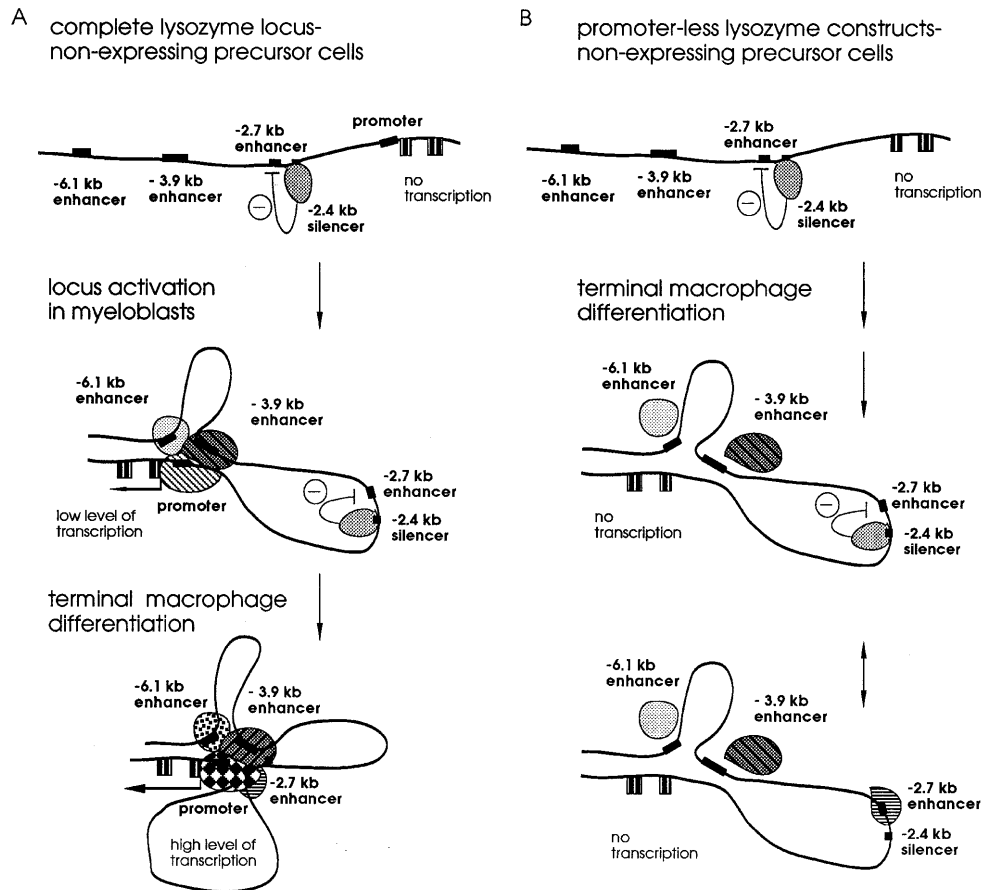


Figure 5. Model of locus activation in the intact and promoter-less lysozyme locus. The 5'-regulatory region of the lysozyme locus is indicated by a line with the black boxes marking the locations of the *cis*-regulatory elements and the striped boxes indicating the position of the first two exons. DNase I hypersensitive sites forming at the various indicated cell differentiation stages are depicted as differentially patterned shapes which indicate transcription factor complexes, which may change their composition during cell differentiation. We assume that transcription factor complexes form at the different enhancer elements and interact with the intact promoter region. (A) Chromatin reorganization of the wt-lysozyme locus; (B) chromatin reorganization of promoter-less constructs. (A and B) Upper panel: structure of the lysozyme locus in lysozyme non-expressing cells. Only the -2.4 kb DHS is formed. (A) Middle panel: Structure of the lysozyme locus in myeloblasts with a low level of lysozyme gene expression symbolized by the horizontal arrow. The early enhancers and the promoter display an active chromatin conformation. The -2.7 kb enhancer exhibits no DHS and the -2.4 silencer is still hypersensitive. (A) Lowest panel and (B) middle and low panel: structure of the lysozyme locus in terminally differentiated macrophages. In terminally differentiated macrophages all *cis*-element regions display an active chromatin conformation and the gene is expressed at its maximal level [in (A) symbolized by the large horizontal arrow]. The two-pointed arrow between the middle and the low panel in (B) depicts the possibility of transitions (or cell population heterogeneities) between an activated chromatin structure at the -2.7 kb E—but less stable than in the intact locus—and the inactive conformation with a DHS at the -2.4 kb S element.

DISCUSSION

Chromatin reconfiguration at the early enhancers requires promoter elements

The deletion of promoter sequences uncovered a difference in the intrinsic ability of the individual *cis*-elements of the chicken lysozyme locus to reorganize chromatin. Our earlier experiments demonstrated that the cooperation of the various *cis*-regulatory regions of the chicken lysozyme gene is essential for its proper transcriptional regulation (51). Investigation of the time course of transcriptional activation of deletion mutants of the lysozyme locus in developing macrophage precursor cells of transgenic mice (53) demonstrated that the early enhancer elements together with the promoter are responsible for its transcriptional activation at early differentiation stages. Our results now demonstrate that these elements are unable to establish an open chromatin structure by themselves, they have to interact with the promoter, most

likely by direct physical contact, as proposed in Figure 5A. Chicken β -globin gene constructs in transgenic mice lacking a promoter exhibit a similar inability to direct DHS formation at the remaining enhancer/LCR. Only when transgene RNA was detectable, probably originating from an adjacent mouse promoter, the DHS appeared (26). A direct physical interaction between enhancer/LCR and promoter elements was suggested to be essential for chromatin reconfiguration and locus activation. Our data support this idea. In our case the results are unambiguous, since we could exclude transcription from an outside promoter, probably because of the presence of the insulating flanking sequences of the complete lysozyme locus.

The MNase generated cleavage patterns at each *cis*-regulatory region of the lysozyme locus in lysozyme non-expressing embryonic fibroblasts of P- mice and XS.0b mice are identical (15), indicating that the promoter deletion does not affect the general chromatin organization of the locus. Although macrophage

stage-specific transcription factors are undoubtedly present in P-macrophages, they are not able to stably bind to their specific recognition sites at the early enhancer elements in promoter deficient constructs. It is possible that transient interactions occur which, however, do not lead to the formation of DHSs due to the absence of stabilizing interactions with promoter elements. A second possibility would be that the lack of an entire promoter with its upstream binding sites and recruited factors abolishes the action of a general chromatin remodeling machine, for example SWI/SNF (62–64). It will be very interesting to determine which promoter sequences are necessary for the formation of a DHS at the early enhancers.

The –2.4 kb silencer and the –2.7 kb enhancer are able to reorganize chromatin in the absence of a promoter

The –2.4 kb silencer is inactive in mature, lysozyme expressing macrophages and is active in all other cell types analyzed. The silencer element extends from –2310 to –2410 bp and carries binding sites for two different proteins. The 3'-site (F2) is a recognition sequence for thyroid (TR) or retinoic acid (RXR) hormone receptors and the 5'-site (F1) is recognized by the chicken homologue of factor CTCF (NeP1) (43,65–67). Our transgenic mouse experiments now show that the silencer element is capable of forming a DHS in any cell type, irrespective of the presence of a promoter. To our surprise, in macrophages of P- mice not only the –2.4 kb DHS but also the –2.7 kb DHS were formed. The nucleosomal organization of the entire –2.4 kb/–2.7 kb region is remodeled towards the potentially active conformation, not only at the enhancer, but also at the silencer element. This type of chromatin rearrangement is normally correlated with maximal transcriptional activity of the lysozyme gene at late macrophage differentiation stages. We have previously shown that the presence or absence of the silencer element has no influence on the time course of activation of the chicken lysozyme gene in developing macrophages (53). We interpreted these results such that this element most likely is repressing the activity of the –2.7 kb enhancer at early stages of macrophage differentiation. Based on the result presented here, we speculate that the autonomous DHS-forming capacity of the silencer element creates the structural prerequisites for the promoter independent DHS formation at the –2.7 kb enhancer. In both chicken and mouse macrophages, the increase in MNase and DNase I accessibility at the enhancer parallels a decrease in accessibility at the silencer (15,48,49). Both elements are located on adjacent positioned nucleosomes (15), whereby the factor binding sites possibly face the same nucleosomal side. Such a spatial arrangement suggests that silencer and enhancer are an integrated *cis*-regulatory element, with factor binding at both sub-elements being mutually exclusive (Fig. 5). DNA bending, shown to be mediated by the active silencer complex (NeP1, TR and/or RXR) on this site (66), may influence nucleosome phasing and thus the spatial arrangement of the regulatory elements. The TR/RXR heterodimer is able to bind its recognition sites (TRE) within chromatin and to repress or activate transcription in the absence or presence of thyroid hormone (TH) (68). However, since the chromatin reorganization in the –2.4 kb/–2.7 kb region is cell differentiation dependent and can be induced in cultured cells solely by induction with LPS (49,60), we assume that it is independent of the presence of a ligand and is at least partially driven by newly synthesized transcription factors recognizing the enhancer element.

The LPS-induced chromatin reorganization in the –2.4 kb/–2.7 kb region seen with the wild-type locus (60) was not observed in P-macrophages. For this aspect of chromatin remodeling the presence of promoter- and/or active upstream enhancer elements is required. In addition, the MNase pattern in non-stimulated cells is generated by a mixture of reorganized and non-reorganized loci. This phenomenon is also seen in the chromatin of mice carrying an enhancer deletion mutant of the lysozyme locus and is caused by genomic position effects (15). Loss of promoter contacts and/or interactions with other *cis*-elements may lead to a reduced probability of establishing a stable –2.7 kb enhancer-complex marked as DHS.

The chicken lysozyme locus does not contain a single element with dominant chromatin opening activity

Ours as well as other studies show that a crucial aspect of locus activation is the ability to open chromatin and to maintain its accessible structure, in order to establish stable gene expression (34,36,69,70). Dominant chromatin-opening activity of one DHS, which would initiate chromatin rearrangements that would spread and permit *trans*-acting factors access to other DHS, has been suggested for hypersensitive site 3 in the human β -globin LCR (34), albeit other results are contradictory (71). However, none of these studies investigated the role of the promoter in this process. In the chicken β -globin locus the presence of an active promoter is essential for DHS formation at the 3' enhancer/LCR (26). A different type of study presented strong evidence that the human β -globin LCR forms a holocomplex with the promoter (34–36,72). Moreover, the human β -globin LCR also consists of functionally interacting components, since removal of one component or exchange of the coding region can abrogate its proper function (34,36,73,74). Our experiments demonstrate that the chicken lysozyme locus harbors no single element with dominant chromatin opening function. Although an element exists which is able to stably reconfigure chromatin in the absence of promoter elements, it acts later in cell differentiation and its chromatin reorganizing capacity is limited to its site. Stable locus activation is mediated by the interplay of separate *cis*-regulatory elements with distinct abilities to generate or maintain transcription competent chromatin structures. Our experiments support the concept that all essential *cis*-regulatory elements—enhancer and promoter elements—have to be integrated into one functional entity to perform locus activation.

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