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 Jägle+, 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 late enhancer appears only later in differentiation, at the promacrophage 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 pIIIilys P–**

The promoter-less lysozyme locus (pIIIilys 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 SalI fragment (S3–S4) from pIIIilys (37) was inserted into the juxtaposed SalI of pIIIiUTR and named pIIIiP-S3S4. In a third cloning step an SpII–XhoI fragment (Sp2–X4) from poly IIIilys was cloned into pIIIiUTR cleaved with SpII and XhoI (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 pIIIilys were cleaved with SpeI. The SpeI fragment in pIIIilys 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 homozgyous progeny. Transgenic mouse lines carrying construct XS (51) were kept as homozgyous 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 10% fetal calf serum (FCS) 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′-CACAAGACTAGAACCCTGC-3′; 5′-GCTGTTGAAAGGACCTCT-3′; mouse-lysozyme (m-lys): 5′-ACCCAGCCTCCAGTACCAT-3′; chicken-lysozyme (c-lys): 5′-GATGCTGCAGGATGAAACGCC-3′; 5′-CTCACAGCAGGCAGCCTTGAT-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 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% PAGE. Gels were stained with ethidium bromide 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, 0.2 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 × 10⁷ to 1 × 10⁸ 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, 2 mM EDTA, 0.2 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.

**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 2–5). 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.
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). The formation of a DHS at the −2.4 kb enhancer is unaffected, an observation which confirms preliminary experiments performed in stably transfected cell culture cells (61). The DHS at the −2.7 kb enhancer 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 enhancer 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– mouse lines (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 and at −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 reprobed 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– mouse 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.

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 reprobed 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 probes, 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.

Figure 4. 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 reprobed 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 probes, 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.
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.
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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