Role of Positive and Negative Cis-regulatory Elements in the Transcriptional Activation of the Lysozyme Locus in Developing Macrophages of Transgenic Mice

(Received for publication, October 9, 1996, and in revised form, November 22, 1996)

Ulrike Jägle‡, Albrecht M. Müller§, Hubertus Kohler§, and Constanze Bonifer¶†

From the Institut für Biologie III der Universität Freiburg, Schänzlestraße 1, D-79104 Freiburg and §Max Planck Institut für Immunobiologie, Stützweg 51, 79108 Freiburg, Federal Republic of Germany

Expression of the chicken lysozyme locus in macrophages is regulated by at least six different positive and negative cis-regulatory elements. Chromatin of the chicken lysozyme locus is gradually reorganized during macrophage differentiation, indicating that each cis-regulatory element is activated at a different developmental stage. Irrespective of their differential developmental activation, individual cis-regulatory regions are capable of driving transcription of the lysozyme gene in mature macrophages of transgenic mice. In order to examine the role of different cis-regulatory regions in lysozyme locus activation, we analyzed the time course of transcriptional up-regulation of deletion mutants of the lysozyme locus in a new in vitro differentiation system based on enriched primary macrophage precursor cells from the bone marrow of transgenic mice. We show that constructs carrying cis-regulatory elements which are structurally reorganized early in development are also transcriptionally active at an early stage. A construct in which the early enhancer has been deleted shows a delay in transcriptional activation. The presence or absence of a negative regulatory element has no influence on the time course of transcriptional activation of the lysozyme locus.

Most gene loci examined so far are regulated by a variety of different cis-regulatory elements distributed over many kilobases of DNA. The presence of a DNase I-hypersensitive site (DHS) in chromatin is the result of the assembly of transcription factor complexes; hence, a DHS in most cases indicates the presence of an active cis-regulatory element. Many gene loci exhibit changes in DHS patterns according to the developmental stage, indicating that along with cellular differentiation, individual cis-regulatory elements of a given gene locus display different activity patterns. It has been argued that a reorganization of chromatin at early stages of cellular differentiation may represent a priming step required for the assembly of an active transcription machinery at later stages of development (1–4). However, the hierarchical relationship between the structural reorganization of cis-regulatory elements and their actual ability to stimulate mRNA synthesis is still unclear (1, 2, 5–7). The dissection of the role of different cis-regulatory elements in the developmental control of gene locus activation requires their individual analysis in an experimental system where cell differentiation can be followed, thus enabling to link a stage-specific chromatin structure with the transcriptional activity of the gene.

We have been studying the chicken lysozyme locus as a marker for macrophage differentiation. Cis-regulatory elements regulating gene expression are located in the 5′-half of the gene locus. Transfection analysis revealed three enhancers located 6.1, 3.9, and 2.7 kb upstream of the transcriptional start site, a hormone responsive element at −1.9 kb, and a complex promoter (8–14). A negative regulatory element is located at −2.4 kb (−2.4-kb silencer), which has been implicated in the repression of the lysozyme locus in lysozyme non-expressing tissues (11, 15, 16). The activity of each of these cis-regulatory elements is marked by the presence of DHS in chromatin (17–20). In turn, reporter gene constructs carrying particular cis-regulatory elements are only active when transfected into cell types displaying a DHS at the position of the same element (13, 14, 19). The analysis of lysozyme chromatin structure in retrovirally transformed chicken cell lines resembling multipotent myeloid progenitor cells (21) and various macrophage maturation stages revealed that different sets of cis-regulatory elements are structurally reorganized at different developmental stages and thus are differentially active (20, 22). Multipotent progenitor cells do not transcribe the lysozyme gene and display the chromatin structure of the inactive locus. At the myeloblast stage, DHs are formed at the −6.1- and the −3.9-kb (early) enhancers as well as at the promoter form. The silencer element is still active, as indicated by the presence of a DHS, whereas the (late) enhancer at −2.7 kb displays no DHS and thus is inactive at this differentiation stage. A low level of lysozyme mRNA can be measured. At later stages of differentiation, a switch in chromatin structure in the region between −2.7 and −2.4 kb is observed. The DHS at the −2.4-kb element disappears, until it is lost upon terminal differentiation and a new DHS at the −2.7-kb enhancer element is formed. Along with chromatin reorganization, transcription of the gene is up-regulated from a very low level in myeloblast-like cells to an almost 100-fold higher level in activated macrophage-like cells. These experiments correlate a high transcriptional level of the lysozyme gene with conditions where all enhancers are active and where the silencer element has been inactivated.

*This work was supported by a grant from the Deutsche Forschungsgemeinschaft (to C.B.) and by the Max Planck Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed. Tel.: 49-761-203-2761; Fax: 49-761-203-2745; E-mail: bonifer@sun2.ruf.uni-freiburg.de.

§The abbreviations used are: DHS, DNase I-hypersensitive site; mAb, monoclonal antibody; LPS, bacterial lipopolysaccharide; HPRT, hypoxanthine phosphoribosyltransferase; kb, kilobase pair(s); PBS, phosphate-buffered saline; FCS, fetal calf serum; RT-PCR, reverse transcription-polymerase chain reaction; M-CSF, macrophage colony-stimulating factor; IL-3, interleukin-3; c-lys, chicken lysozyme; m-lys, mouse lysozyme; GM-CFCs, granulocyte macrophage-colony forming cells; M-CFC, macrophage-colony forming cells.

¶M. C. Huber, G. Krüger, and C. Bonifer, unpublished observations.
We showed that the chromatin structure displayed by the lysozyme locus in the various chicken cell types is faithfully reformed in lysozyme expressing and non-expressing cells of transgenic mice. This holds true for the DHS and the nucleosomal spacing pattern as well as for the reorganization of the −2.4/−2.7-kb region after terminal macrophage differentiation (23, 24), demonstrating that the same chromatin rearrangements take place in both species. Our experiments in transgenic mice also showed that for position-independent expression of the lysozyme locus in mature macrophages, the presence of all cis-regulatory elements is necessary (25, 26). However, experiments with deletion mutants of the lysozyme locus in transgenic mice demonstrated that each enhancer region, despite their unique temporal regulation, is capable of driving macrophage-specific expression in mature macrophages (26). The question now remained of how the different cis-regulatory elements cooperate during earlier stages of cell differentiation. To this end, we have analyzed the time course of transcriptional activation of wild type and mutant lysozyme locus constructs during in vitro differentiation of myeloid precursor cells isolated from the bone marrow of transgenic mice. We show that constructs carrying a deletion of the late enhancer region including the silencer element are transcriptionally activated at the same developmental stage as the wild type locus carrying all cis-regulatory elements. The time course of activation of both constructs is indistinguishable, indicating a coincidence of structural reorganization of the early enhancers and the onset of mRNA synthesis. A construct in which the early enhancer has been deleted shows a delay in transcriptional activation. Our results suggest that the presence of the −2.4-kb silencer element on the wild type locus has no influence of the developmental onset of lysozyme locus activation, which is solely determined by the activity of the early enhancers.

EXPERIMENTAL PROCEDURES

Immunofluorescence Labeling and Cell Sorting—For each experiment four mice were killed by cervical dislocation. Bone marrow cells were collected from femurs and tibiae, washed twice in phosphate-buffered saline (PBS). Cells were collected, washed once in PBS, and maintained on ice throughout the staining procedure. The cell suspension was depleted of red blood cells by hypotonic lysis with an ammonium chloride potassium buffer (Red Cell Lysis Buffer, Sigma). To remove debris, the suspension was centrifuged through a cushion of fetal calf serum (FCS; Life Technologies, Inc.). The remaining cells were washed once and resuspended in PBS supplemented with 3% FCS. After the suspension was passed through a cell strainer (Falcon/Becton Dickinson), 3.5 × 10⁶ cells in a volume of 200 µl were used for staining. To suppress nonspecific binding, cells were first incubated for 5 min with 15 µl of rat serum. Afterwards cells were incubated for 15 min with 16 µl of biotinylated ER-MP12/M-3 (BMA), followed by an addition of 16 µl of streptavidin-Red670 and another incubation of 15 min. After two washes, the cells were resuspended in PBS, 3% FCS to a final concentration of 2 × 10⁶ cells/ml. Stained cells were analyzed and sorted on a FACSStar™ (Becton Dickinson). The fluorescence intensity of individual cells was measured as relative fluorescence units. The purity of the sorted cell populations was determined by flow cytometry and exceeded 95%. In order to characterize cells differentiated in vitro by antibody staining, cells were stained with ER-MP12 and ER-MP20 as described above. In addition, cells were stained with R-phycocerythrin-coupled mAbs (Pharmpingen) against B220 (RA3–6B2), Mac-1 (M1/70), F4/80 and IgM (isotype control). Stained cells were analyzed by flow cytometry.

IL-3- and M-CSF-stimulated Culture of Sorted Cell Populations—The medium used for the culture of sorted cells was IMDM (Life Technologies, Inc.) supplemented with 10% FCS, 5% conditioned medium containing IL-3, 10% L-cell conditioned medium containing M-CSF, 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin (Life Technologies, Inc.), and 1.5 × 10⁻⁶ M monothioglycerol. Conditioned media were prepared as described (27). After sorting the cell populations ER-MP12/ER-MP20 and ER-MP12/ER-MP20 were distributed into 24-well plates (Greiner) depending on the number of kinetic time points and were cultured in a humidified environment with 5% CO₂ in air at a temperature of 37 °C. Depending on the sorting efficiency 1.5–3 × 10⁶ ER-MP12/ER-MP20 and 8–10 × 10⁶ ER-MP12/M-3 cells were plated per well. When harvesting the cells in intervals of either 12 or 24 h, the adherent cell fractions were separated from the non-adherent fractions. When indicated, cells were stimulated with 5 µg/ml LPS for 12 h. In order to obtain cells for antibody staining, cells were plated onto hydrophobic Petri dish cell culture dishes (Heraeus). Test experiments indicated that no difference in gene expression was found as compared with normal tissue culture dishes (data not shown).

RNA Isolation and cDNA Preparation—Total RNA was isolated from the adherent and non-adherent cells using 0.5 ml of RNAzol™ B (Biotex 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 (Life Technologies, Inc.) in a reaction volume of 20 µl under conditions recommended by the manufacturer. PCR reactions were carried out in a Trio-Thermoblock (Biometra) using a regimen of 1.5 units of Taq polymerase (Life Technologies, Inc.), PCRs were performed with 1.25 mM MgCl₂ and 15 pmol of each m-lys primer, whereas chicken lysozyme used 1.25 mM MgCl₂ and 18 pmol of each HPRT primer. PCRs for mouse lysozyme used 1.25 mM MgCl₂ and 15 pmol of each m-lys primer, whereas chicken lysozyme PCRs were performed with 1.0 mM MgCl₂ and 15 pmol of each m-lys primer. To every PCR reaction 1 µl of the following serial dilutions from the determined dilution of every sample which gave rise to a linear PCR product was added to analyze the different mouse lines: mouse line XS: 0.1, 1.2, 1.1, 1.5; mouse line XDS/SS: 2.1, 2.4, 2, 1.5. For mouse line dXK, 2.4, 2, and 1 µl were used. Primers used were as follows: HPRT, 5′-CACAGGCTAGAACACCTGC-3′; 5′-GCTGGTA-

RESULTS

Experimental Strategy—The different cis-regulatory elements of the lysozyme gene are structurally activated at different macrophage differentiation stages as indicated by a changing pattern of DHS in the 5′-regulatory region of the lysozyme locus in various myeloid cell lines (20) (Fig. IA). The highest transcriptional level is observed in cell lines...
representing activated macrophages that display DNase I hypersensitivity at the position of all enhancer elements and where the DHS at the silencer element at \(2.4\) kb has completely disappeared. In order to evaluate the contribution of different cis-regulatory region to the developmental control of lysozyme expression, we analyzed the time course of transcriptional activation in the three different mouse lines indicated in Fig. 1, B and C (26). Mouse line XS.0b carries a construct with the full set of cis-regulatory elements, whereas the transgenes in mouse lines dXK.2 and XSdSS.28 carry either a deletion of the early enhancer region around \(2.6.1\) kb or a deletion of the late \(2.2.7-2.4\) kb enhancer region, respectively. The presence or absence of domain bordering sequences has no influence on the expression of the different constructs in macrophages, as shown earlier (26).

In order to follow transcriptional activation of wild type and mutant lysozyme loci, we set up an in vitro differentiation system based on enriched myeloid precursor cells as outlined in Fig. 2. It has been shown that developing macrophage precursors can be characterized by a differential expression of the ER-MP12 and the ER-MP20 surface antigens (28, 29). The most immature macrophage colony-stimulating factor (M-CSF) responsive precursors express a high level of the ER-MP12 and no ER-MP20 antigen (Fig. 2). During macrophage maturation, ER-MP12 expression is gradually switched off, whereas expression of ER-MP20 is switched on. Bone marrow monocytes express a high level of the ER-MP20 antigen and have lost the ER-MP12 antigen (30). We isolated ER-MP12\(^{hi}\)/20\(^{lo}\) cells (ER-MP12\(^{hi}\) population) by fluorescence activated cell sorting in order to follow gene expression during early macrophage differentiation. To compare the mRNA levels in monocytes differentiated in vitro with the level found in bone marrow monocytes, we also analyzed ER-MP12\(^{lo}/20^{hi}\) cells (ER-MP20\(^{hi}\) population). The isolated cell populations were cultivated under macrophage growth promoting conditions in the presence of M-CSF and interleukin-3 (IL-3) for a period of 6 days. Bacterial lipopolysaccharide (LPS) is known to promote macrophage activation and has been shown to stimulate expression of the chicken lysozyme gene in chicken and mouse macrophages (10, 20, 27, 31). To examine at which stage of differentiation the cells acquire LPS responsiveness, cultures were treated with LPS for 12 h, either immediately after isolation or after 5 days of culture. Along with progressive macrophage maturation the cells acquire an adherent phenotype. Since we were interested in studying gene activation starting with a precursor population as immature as possible, we separated the cells into more mature adherent and immature non-adherent fractions. Cells were harvested at 12- or 24-h intervals as indicated in Fig. 2, and total RNA was prepared which was subjected to RT-PCR analysis for the expression of the following genes: HPRT as an internal calibration standard, the endogenous macrophage-specific mouse lysozyme M gene as a control marker for a successful and reproducible in vitro differentiation, and the chicken lysozyme transgene. In order to define the developmental stage at which transgene activation takes place, we immunostained cells at various time points of differentiation with labeled antibodies directed against markers indicative of different macrophage maturation stages (32).
Characterization of in Vitro Differentiated Myeloid Cells—Macrophages developing from ER-MP12hi populations in vitro have up to now not been characterized in detail. The ER-MP12hi population consists mainly of small blast-like cells as described previously (30, data not shown). From day 3 of culture onwards, cells progressively acquire an adherent phenotype (see also Fig. 3). By day 6 most cells are adherent and from morphology and surface marker expression (Fig. 5) resemble monocytes. The ER-MP20hi population resembles monocyctic cells, which adhere quickly within 12 h of culture (see Fig. 3). During progressive differentiation of both populations acquire the characteristic features of macrophages, like irregular shapes and granules.

In order to further characterize cells developing in our culture system, we analyzed their proliferative capacity. In each respective differentiation culture we started with the same number of cells; hence, the comparison of the total amount of HPRT mRNA present in our RNA preparations from the different time points allowed an accurate description of relative cell numbers. In the same way, we could measure the proportion of adherent versus non-adherent cells. The result of this experiment is depicted in Fig. 3, which shows the relative increase in cell numbers and the changing proportion of adherent versus non-adherent cells in the different cell populations. An initial lag phase is observed for the ER-MP12hi culture but not of mouse lysozyme mRNA levels. A similar phenomenon is observed with the differentiated ER-MP20hi cell population, where transgene expression is initially high (probably as a result of activation via Fc receptors) and sharply declines after a couple of days in culture. However, in both populations LPS treatment leads to a reactivation of transgene expression.

Transcriptional Activation of the Wild Type Chicken Lysozyme Locus during in Vitro Differentiation—Construct XS carries the full set of cis-regulatory elements of the lysozyme locus and is expressed at a high level in macrophages of transgenic mice and independent of the chromosomal position of the transgene (26). Macrophage precursor cells from the bone marrow of transgenic mouse line XS.0b were isolated as described above and subjected to in vitro differentiation, and RNA prepared from the various cell populations was analyzed by RT-PCR. Since the ER-MP20hi cell population is composed almost entirely of adherent monocytes, only the adherent cell fraction was examined. Fig. 4 shows the results of this experiment. One example for such an RT-PCR analysis is depicted in Fig. 4A. The time course of transcriptional activation of transgene and endogenous lysozyme gene was determined by quantifying PCR signals derived from the linear part of the amplification reaction (Fig. 4B) for each time point.

Expression analysis for chicken lysozyme and mouse lysozyme expression in the ER-MP12hi fraction at day 0 revealed a very low level of expression of both genes. Expression of the chicken lysozyme transgene in the differentiating non-adherent fraction linearly increases from day 0.5 of in vitro differentiation onwards, whereas expression of the endogenous mouse lysozyme gene increases exponentially. The adherent ER-MP12hi population shows a different time course of expression. Initially, both transgene and mouse lysozyme mRNA levels increase during differentiation culture. Surprisingly, progressive differentiation in culture leads to a decrease of transgene but not of mouse lysozyme mRNA levels. A similar phenomenon is observed with the differentiated ER-MP20hi cell population, where transgene expression is initially high (probably as a result of activation via Fc receptors) and sharply declines after a couple of days in culture. However, in both populations LPS treatment leads to a reactivation of transgene expression.

For comparison, we measured the chicken lysozyme expression level in thioglycolate elicited, relatively mature peritoneal macrophages of the same mouse line. The mRNA level in these cells that had been cultured for 1 day was comparable to the highest level found in in vitro differentiated cells but did not decrease during prolonged cell culture (data not shown). Only the more mature ER-MP20hi population but not the non-adherent or the adherent ER-MP12hi cell population responds to LPS treatment at early time points of differentiation. Expression of the endogenous mouse lysozyme gene was not induced by LPS treatment, as shown before (27, 31).
FIG. 4. Developmental activation of construct XS carrying the full set of cis-regulatory elements. A, expression of the chicken lysozyme transgene (c-lys), the endogenous mouse lysozyme gene (m-lys), and the HPRT gene in the non-adherent (ER-MP12\(^{hi}\) na) and the adherent (ER-MP12\(^{hi}\) a) fraction of the ER-MP12\(^{hi}\)/20\(^{hi}\) cell population as well as from the adherent fraction (ER-MP20\(^{hi}\) a) of the ER-MP12/20\(^{hi}\) cell population. Selected RT-PCR experiments using high cDNA concentrations are shown which emphasize early stages of differentiation where transgene and mouse lysozyme expression levels are low. Note, therefore, that the PCR signals at later differentiation stages are outside the linear range of the amplification reaction and are shown only for reasons of completeness. B, quantification of PCR signals. Three different cDNA concentrations were used for the PCR reaction to ensure that amplification was in the linear range. Bands were densitometrically scanned as described under “Experimental Procedures,” the resulting signals specific for c-lys (upper panel) and m-lys (lower panel) were normalized for RNA variation against the HPRT signal and plotted against culture duration. Curves were calculated with a Kaleidograph program on a Macintosh computer. The plot depicts mean values (where possible) of two independently performed but overlapping kinetics. Cells were harvested either every 12 h over a period of 3 days or harvested every 24 h over a period of 6 days. Open circles and squares, cells of the freshly sorted cell population that had not been separated by selective adherence. Dotted circles and squares, cells stimulated with LPS for 12 h.

Analysis of in Vitro Differentiating Cells—In order to determine the differentiation stage in which the chicken lysozyme transgene is activated in mouse cells, we analyzed the surface marker expression of differentiating cells. We stained cells at various time points of in vitro differentiation with antibodies directed against the ER-MP12 and ER-MP20 antigens, as well as against CD11b (Mac-1) (33) and F4/80 (34), the latter being surface markers characteristic for mature macrophages. A variant of the common leukocyte-specific antigen CD45R is expressed predominantly in B-cells and a subset of macrophages (35) and is recognized by the B220 antibody. An antibody against mouse IgM served as isotype-matched control (36). The results of these analyses are shown in Fig. 5. As predicted, in vitro differentiation of the ER-MP12\(^{hi}\) population leads to a successive down-regulation of the expression of the ER-MP12 antigen and to an up-regulation of the ER-MP20 antigen (30). The cells reach the double positive stage around days 2–3 of differentiation, about 8% of all cells display this phenotype at day 3 (data not shown). Subsequently, the cells express more and more epitopes characteristic for mature macrophages. Initially the expression of the B220 antigen is high, indicative of the presence of B-cell precursors in this population (37). Stimulated development of macrophages leads to a decrease in the number of B220 positive cells. The ER-MP20\(^{hi}\) population is characterized by a high level of expression of antigens specific for mature macrophages. Taken together, our analysis shows that chicken and mouse lysozyme gene expression is up-regulated from day 0.5 of differentiation onwards, where the cells have not yet reached the ER-MP12/ER-MP20 double positive stage and express only a low level of mature macrophage surface markers.

The Early Enhancer Region Is Responsible for the Activation of the Lysozyme Locus at Early Stages of Development—We analyzed the time course of transcriptional activation of two different deletion mutants of the lysozyme locus during in vitro differentiation (26). Precursor cells of mouse lines XSdSS.28 and dXK.2 (Fig. 1) were isolated, and RNA from in vitro differentiated cells was analyzed by RT-PCR as described for mouse line XS.0b. Fig. 6, A and B, displays the results obtained for mouse line XSdSS.28, which carries a construct lacking the late enhancer region. The kinetics of transcriptional activation of this construct is undistinguishable from the construct carrying all cis-regulatory elements (Fig. 4). Here also we observe a continuous increase of expression in immature precursors followed by a decrease in more mature cell populations. The kinetics of transcriptional activation of construct dXK which lacks the −6.1-kb enhancer region was different (Fig. 6, C and D). Expression in freshly isolated ER-MP12\(^{hi}\) cells at day 0 was high, probably as a result of contaminating more mature cells, which were lost due to their adherence after 1 day in the differentiation medium. Note that these contaminating cells also have already acquired LPS responsiveness (Fig. 6C, ER-MP12\(^{hi}\) a). Expression in precursor cells did not increase until day 2.5 of differentiation and thereafter was up-regulated with
Fig. 5. Immunophenotypic characterization of differentiating cells. Cell surface marker characterization of ER-MP12\(^{hi/20^-}\) (A) and ER-MP20\(^{hi/12^-}\) cells (B) during their in vitro differentiation in the presence of IL-3 and M-CSF and unseparated bone marrow (BM, upper panel,
a kinetics similar to that observed for the other constructs. As with the other constructs, expression in the ER-MP20\(^\text{hi}\) cell population was high in the beginning but decreased toward terminal differentiation of the cells (Fig. 6, C and D, ER-MP20\(^\text{hi}\) a). Expression from the XSdSS\(^2\) and most likely also from the dXK construct was refractory to LPS stimulation at early differentiation stages but could be induced at later stages as it has been shown before (31). This holds true for all cell populations analyzed. The time course of activation of the endogenous mouse lysozyme M gene in all in vitro differentiation cultures was basically indistinguishable, indicating that the time course of transgene activation is a function of the various constructs analyzed and is not due to differences in the differentiation kinetics of the cells.

**DISCUSSION**

*The Wild Type Chicken Lysozyme Locus Is Transcriptionally Activated in Mouse Macrophages at the Same Developmental Stage as in Chicken Macrophages*—In our in vitro differentiation experiments we analyzed two populations of macrophage precursor cells representing two different maturation stages. Previous studies have shown that the ER-MP12\(^{2h}/20^\text{th}\) population contains about 50% morphologically undifferentiated blast cells, and the other half represents identifiable blast cells of the myeloid, the lymphoid, and the erythroid lineage (30, 38). 12% of the total cell population represents a mixture of granulocyte macrophage-colony forming cells (GM-CFCs) and macrophage-colony forming cells (M-CFCs/monoblasts) with a high proliferative potential (30). The heterogeneous composition of this population explains the apparent lag phase in proliferation, since precursor cells of other hematopoietic lineages are unable to proliferate under our culture conditions. However, since we observe no reduction of relative cell numbers, we assume that myeloid precursors start proliferating immediately after plating. The ER-MP12\(^{2h}/20^\text{th}\) population contains predominantly monocyctic cells (74%) with a few immature blasts cells (Ref. 30 and this study), which both immediately start to proliferate. Our experiments clearly demonstrate that under our culture conditions both sorted cell populations proliferate and simultaneously differentiate. The ER-MP12\(^{2h}/20^\text{th}\) population reaches the differentiation state of the ER-MP12\(^{2h}/20^\text{th}\) population around day 6 as judged from mouse lysozyme and surface marker expression levels as well as from the ratio of adherent to non-adherent cells.

Our three-color surface marker analyses of differentiating ER-MP12\(^{2h}/20^\text{th}\) cells indicate that they transiently mature

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3 M. de Bruijn and P. Leenen, personal communication.
into cells expressing both the ER-MP12 and the ER-MP20 antigen around days 2–3 of in vitro differentiation. A cell population isolated from the bone marrow expressing this surface marker combination has been shown to consist of some M-CFCs and promonocytes (30). The increase in chicken lysozyme expression begins at day 0.5 of differentiation whereby half-maximal expression is reached by day 3. The onset of transgene expression occurs therefore most likely at the GM-CFC stage (which is analogous to the myeloblast stage in the chicken system), indicating a concordance in developmental regulation between the two species (20). Since also the same developmentally controlled chromatin rearrangements occur in both species, we were able to draw relevant conclusions from the analysis of wild type and mutant gene loci in transgenic mice.

Transgene expression decreased at later macrophage differentiation stages. This phenomenon was observed for all constructs tested. However, it was not observed with thioglycolate-elicited peritoneal macrophages kept in culture for up to 2 weeks, indicating that the lysozyme transgene is expressed at a high level in in vitro differentiated cells. The uniformly high expression level of the endogenous mouse lysozyme gene as well as the reactivation of a high level of transgene expression by LPS treatment argues against a general decrease of cell viability in our culture system. It rather indicates that the cells reach a quiescent differentiation stage which does not support transgene expression without a further signal. In vivo, monocytes migrate into various tissues and differentiate into various mature macrophage types. In most cases such cells also cease expression of the endogenous mouse lysozyme gene. We could show that expression of the chicken lysozyme transgene, in contrast to the mouse lysozyme gene, is a faithful marker for macrophage activation (27, 31). Our experiments indicate that transgene expression is up-regulated until the cells reach the monocyte stage where they normally would leave the bone marrow is then down-regulated and is only reactivated after further macrophage differentiation. Thioglycolate-elicited macrophages obviously have received an additional differentiation signal required to maintain a high transgene expression level, which in addition can be further enhanced by LPS treatment (31).

The Role of the Different Cis-regulatory Elements in Lysozyme Gene Regulation—The various enhancer elements on the lysozyme locus assemble transcription factors and thus become DNase I-hypersensitive at different stages of macrophage development. Our studies of the time course of activation, as well as earlier chromatin structure analyses, point to a different role of various cis-regulatory elements with respect to the coordinated activation of the lysozyme locus during development. In early macrophage precursor cells the early enhancers, the promoter, and the silencer element are active, whereas the late −2.7-kb enhancer is inactive (20, 22, 23). Hence, several scenarios could be envisaged. Either the early (−6.1-kb and −3.9-kb) enhancers and the promoter drive lysozyme transcription at a low frequency irrespective of the presence of the silencer at −2.4 kb or the functional silencer element competes with the enhancers and inhibits transcription at early differentiation stages. This would allow the gene to be transcribed only after the developmentally controlled inactivation of the silencer element and the simultaneous activation of the late −2.7-kb enhancer. The third possibility would be a developmentally controlled reorganization of the early enhancers by transcription factors synthesized later in differentiation which in turn would be necessary for their interaction with the basal transcription machinery. The two latter models would imply that the structural reorganization of the early enhancers is uncoupled from their ability to drive transcription.

Each of these scenarios could be experimentally distinguished. If the two latter models were true, mutants of the lysozyme locus carrying deletions of early or late enhancer regions should have shown characteristic differences in the time course of developmental activation as compared with a locus carrying all cis-regulatory elements. Such differences should also have been observed if the presence of the −2.4-kb silencer element had any influence on the onset of lysozyme expression at early differentiation stages, i.e. if it would repress expression of the lysozyme locus in early precursors. However, the time course of transcriptional activation of the chicken lysozyme transgene, with or without the silencer element, is identical. Both transgenes up-regulate transcription at day 0.5 of in vitro differentiation and decrease expression at later differentiation stages with similar kinetics. This is in contrast to the construct carrying a deletion of the −6.1-kb enhancer region. Here, in concordance to the chromatin studies, we observe a 2-day delay in the onset of transcriptional activation. At day 2.5 to 3 the cells reach the ER-MP12/ER-MP20 double positive (promonocytic) stage. At this stage, in analogy to the chicken system, the −2.7-kb enhancer is reorganized and thus activated. Our results also indicate that the presence of the early −3.9-kb enhancer alone is not sufficient to activate transcription in early progenitors.

Our results demonstrate that the absence of the silencer element does not alter the time course of transcriptional up-regulation, implying that this element does not repress the action of the early enhancers. Although we have no evidence to suggest this from the present analysis, we prefer a model in which the −2.4-kb element is involved in repressing the macrophage-specific −2.7-kb enhancer element at early developmental stages of myeloid differentiation. The same element might also be responsible for the repression of the −2.7-kb enhancer in the chicken oviduct, where the gene is expressed under steroid hormone control. Support for this idea comes from experiments that demonstrate the presence of a DHS at the −2.4-kb element in the chicken oviduct, where the −2.7-kb enhancer is not hypersensitive and thus not active (18). Previous chromatin analyses have demonstrated that the silencer element and the immediately juxtaposed enhancer element are each organized in a positioned nucleosome and may form an integrated cis-regulatory element. The spacing of binding sites is such that they may face the same side on each nucleosome, thus bringing them in close contact (23). Chromatin rearrangements at the −2.4-kb and at the −2.7-kb elements are strictly parallel, and the appearance of MNase and DNase I-hypersensitive sites at the enhancer correlates with the disappearance of such sites at the negative regulatory element, indicating that factor binding at both elements is mutually exclusive.

Taken together our data indicate that initial locus activation is performed by the interaction of the early enhancers with the promoter. Maximal transcriptional activity, which is necessary during a bacterial attack (simulated by LPS treatment), is achieved by the inactivation of the negative regulatory element and the simultaneous activation of the −2.7-kb enhancer. In addition, the activity of all enhancers as well as the promoter can be modulated by LPS and other macrophage activating agents (10; 31; 39), indicating that the expression status of the chicken lysozyme locus, as an endogenous gene in chicken and as a transgene in the mouse, is strongly dependent on the physiological status of a macrophage cell.

Acknowledgments—We are particularly indebted to M. van Bruijn and P. Leenen, Erasmus University of Rotterdam, for generous advice and the availability of information prior to publication. We thank Dr.

4 N. Faust, C. Bonifer, and A. E. Sippel, manuscript in preparation.
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