

The activation of the chicken lysozyme locus in development is a cooperative process

Research Article

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Summary

The chicken lysozyme gene is a marker for the myelomonocytic lineage of the hematopoietic system. In early experiments we demonstrated that correct activation of the chicken lysozyme locus in macrophages of transgenic mice requires the complete set of cis-regulatory elements. Different cis-elements are activated at distinct developmental stages and their chromatin structure is differentially remodelled. We have shown that the early onset of transcriptional activation of the chicken lysozyme locus is entirely dependent on enhancer elements which are structurally activated early in development (-6.1 kb and -3.9 kb early enhancers). However, the structural reorganization of the early enhancers requires the presence of promoter sequences. We concluded from these experiments that the early enhancers and the promoter cooperate in order to activate the lysozyme locus. Subsequently, we performed experiments aimed at elucidating the cis-regulatory requirements of chromatin rearrangement at the early enhancers. The -6.1 kb enhancer is well characterized at the molecular level and all transcription factors contributing to its activity in transfection studies are known. We have placed this element into a new sequence context on the lysozyme locus by deleting extended flanking regions and analyzed this construct in transgenic mice. Surprisingly, its chromatin rearrangement ability as judged from DNaseI hypersensitive site formation was impaired. We conclude from this experiment that the cooperation of enhancer core and flanking sequences is necessary for enhancer activity. We hypothesize that all sequences of a gene locus serve a purpose in the developmental control of its activation.

I. Introduction

One of the key objectives in developmental biology is to understand how the regulatory information from the genome is translated into the controlled expression of different genes. The gradual change in the activity of individual gene loci is the basis for the various steps in the commitment of a differentiating cell towards the terminally differentiated state. A large number of gene expression studies have been performed to describe cellular differentiation processes. However, due to the difficult nature of the experiments involved, the molecular details of cell differentiation at the level of the genome still remain to be elucidated. Eukaryotic genes are regulated by a number of different cis-regulatory elements distributed over large distances. In addition it has been

demonstrated that, depending on the developmental stage, different combinations of transcription factors can occupy the same cis-regulatory element (Roque et al., 1996; Gualdi et al., 1996). It is known that different members of the same transcription factor family can be differentially expressed in development. Thus, some transcription factors will only transiently occupy cis-regulatory elements. At present it is not clear, whether only one type or different factor family members can occupy the same binding site within a larger protein complex at the various developmental stages.

Another open question concerns the order of transcription factor assembly. Many cis-regulatory elements are organized in positioned nucleosomes (Richard -Foy and Hager, 1987; Straka and Hörz, 1991).

Since not all transcription factors are capable of binding when their recognition sequences are organized in a nucleosomal core (Blomquist et al., 1996b; Taylor et al., 1991; Pina et al., 1990) this, in turn, implies that a certain order of factor complex assembly may be necessary for correct regulation. In addition, factors capable of recruiting chromatin modifying enzyme like histone acetylases might have to pave the way for other factors joining the transcription complex later in the assembly process (reviewed in: Grunstein, 1997; Peterson and Tamkun, 1995).

Last, but not least, the role of sequences flanking cis-regulatory elements has been elusive. The cores of cis-regulatory elements are very often separated by long arrays of flanking DNA which on first sight seem to serve no purpose. However, for genes whose role in different species have been conserved, like the globin or the Hox gene clusters, we also find a conservation of gene order and spacing, suggesting a regulatory role of such sequences. It is therefore tempting to speculate that a eukaryotic gene locus is not just a collection of cis-regulatory elements separated by "junk" DNA, but that there is more to it than that.

II. The lysozyme locus as a model for gene locus activation

In order to answer these questions we have been studying the molecular basis of transcriptional activation of the chicken lysozyme locus in the myeloid lineage of the mammalian hematopoietic system. The lysozyme locus is small (21 kb), thus facilitating the manipulation of individual cis-regulatory elements within the context of an entire genomic locus. Expression of the lysozyme gene is regulated by a combination of several cis-regulatory elements located in the 5'-half of the locus. Three enhancers, 6.1kb-, 3.9 kb- and 2.7 kb upstream of the transcriptional start site as well as a silencer element at -2.4 kb and a complex promoter (Baniahmad et al., 1987; Grewal et al., 1992; Hecht et al., 1988; Theisen et al., 1986; Steiner et al., 1987; Baniahmad et al., 1990; Luckow and Schutz, 1989; Sippel et al., 1987a) have been identified. All active cis-regulatory elements colocalize with DNaseI hypersensitive sites (DHSs) in chromatin (Fritton et al., 1984; Fritton et al., 1987; Sippel et al., 1988; Huber et al., 1995; Sippel et al., 1996).

In order to be able to draw relevant conclusions regarding the contribution of each cis-element to lysozyme gene regulation, we first determined in transgenic mice which of several modified constructs was specifically expressed in the right cell type (macrophages) and was unaffected by chromosomal position effects. This holds true for the complete chicken lysozyme locus carrying the full set of regulatory elements (Bonifer et al., 1990). Deletion of one enhancer region abolishes position independence of expression, indicating that for position

independent transgene expression the cooperative action of all cis-regulatory elements is necessary (Bonifer et al., 1994b). Repression of gene expression by genomic position effects is correlated with suppression of DHS formation and with an inefficient reorganization of nucleosomes in the cis-regulatory regions (Huber et al., 1994; Huber et al., 1996), indicating that active chromatin formation and transcriptional activity are closely linked.

The structural activation of the lysozyme locus takes place in several steps. Accordingly, the individual enhancer elements of the lysozyme locus can be categorized into early or late enhancers. The early enhancers at -6.1kb and -3.9kb and the promoter become DNaseI hypersensitive at the myeloblast stage when the gene is also transcriptionally activated. A low level of gene expression is observed. The DHS at the silencer element is still present. The DHS at the late -2.7kb enhancer appears only later in differentiation. Simultaneously, the -2.4kb silencer disappears (Huber et al., 1995). Each cis-regulatory element shows a distinct structural organization, with transcription factor binding sites specifically arranged with respect to nucleosomes. Transcriptional activation leads to a rearrangement of chromatin structure in an element-specific fashion (Huber et al., 1996). The results of our structural studies suggest that the correct alignment of transcription factor binding sites with respect to the position of nucleosomes may be essential for their interaction and thus for position independent expression.

III. The activation of the chicken lysozyme locus in development requires the interaction of a subset of enhancer elements with the promoter

Together with the promoter, each enhancer is capable of activating the gene locus specifically in mature macrophages of transgenic mice (Bonifer et al., 1994b; Jäggle et al., 1997). 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 -6.1kb enhancer leads to a delay in gene activation, indicating that this element plays a crucial role in the initial activation process (Jäggle et al., 1997). The presence of the -3.9 kb enhancer alone is insufficient for the early onset of transcription.

We subsequently examined the role of the promoter in lysozyme locus activation by analyzing a construct carrying the complete lysozyme locus with an internal deletion of the promoter in transgenic mice (Huber et al., 1997). Transcription from this construct was completely abolished. However, the formation of a DHS at the -2.4 kb silencer element was unaffected and in macrophages, the DHS at the -2.7 kb enhancer element was formed. In

contrast, DHS formation and chromatin remodelling at the early -6.1 kb and -3.9 kb enhancers were abolished. Taken together our data indicate that in the initial activation of the lysozyme locus the early enhancers have to interact with the promoter.

How this interaction takes place and which transcription factors are involved is at present unknown. However, since our initial promoter deletion removed defined sequence elements from the complete lysozyme locus without any further changes, we are now in the position to replace defined sequence elements in the promoter and thus analyze transcription factor interaction truly involved in gene locus activation.

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

The -2.4kb silencer is inactive in mature, lysozyme-expressing macrophages and is active in all other cell types analyzed. It extends from -2310bp to -2410bp and carries a recognition sequence for thyroid (TR) or retinoic acid (RXR) hormone receptors. The second identified protein is the chicken homologue of factor CTCF (NeP1) (Köhne et al., 1993; Baniahmad et al., 1990; Arnold et al., 1996; Burcin et al., 1997). Our transgenic mouse experiments now show that the silencer element is capable of forming a DHS in any cell type also in the absence of a promoter. To our surprise, in macrophages of such mice not only the -2.4kb DHS but also the -2.7kb DHS was formed. This type of chromatin rearrangement is normally correlated with maximal transcriptional activity of the lysozyme gene at late macrophage differentiation stages.

We could show 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 (Jäggle et al., 1997). It is therefore possible that this element is repressing the activity of the -2.7kb enhancer at early stages of macrophage differentiation. In both, chicken and mouse macrophages, the increase in MNase- and DNaseI accessibility at the enhancer parallels a decrease in accessibility at the silencer (Huber et al., 1995; Huber et al., 1996; Sippel et al., 1988). Both elements are located on adjacent positioned nucleosomes (Huber et al., 1996), 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. DNA bending, shown to be mediated by the active silencer complex on this site (Arnold et al., 1996), may influence nucleosome phasing and thus the spatial arrangement of the regulatory elements. The TR/RXR heterodimer is able

to bind its recognition sites within chromatin and to repress or activate transcription in the absence or presence of thyroid hormone (TH) (Wong et al., 1995).

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 (Huber et al., 1995; Faust et al., 1997), we assume that it is independent of the presence of a TR/RXR ligand and thus is at least partially driven by transcription factors recognizing the enhancer element in a differentiation dependent fashion. We could indeed demonstrate a LPS stimulated binding of members of the C/EBP transcription factor family to the -2.7 kb enhancer (Faust N, Bonifer C, and Sippel AE, submitted). Whether the formation of the DHS at the silencer element is the structural prerequisite for the promoter-independent DHS formation of the -2.7 kb enhancer or whether the factors binding to the enhancer are capable of disrupting chromatin by themselves remains to be tested.

V. Results and Discussion

A. Enhancer promoter interaction in lysozyme locus activation

Our earlier experiments have demonstrated the existence of two different classes of cis-regulatory elements of the lysozyme locus. The early enhancers at -6.1 kb and -3.9 kb need a promoter in order to remodel chromatin, whereas the silencer element at -2.4 kb and the enhancer at -2.7 kb do not (**Figure 2**). In turn, our functional experiments showed that the correct timing of activation of the lysozyme locus is dependent on the presence of the -6.1 kb enhancer. **Figure 1** depicts our present model of the various cell differentiation dependent regulatory states of the lysozyme locus as deduced from our structural and functional experiments. In our structural experiments we have employed retrovirally-transformed myeloid cell lines representing various fixed differentiation states.

In differentiating primary cells we envisage the structural reorganization of the complete lysozyme locus as a dynamic process which involves the assembly and cooperative action of a large number of transcription factors as well as chromatin modifying enzymes. In order to understand the molecular details of this process we have to fully characterize the transcription factor composition of each cis regulatory element. However, here we encounter a problem: as with most other gene loci, most cis-regulatory elements of the lysozyme locus have been defined and analyzed the classical way, that is, by transient transfection assays as well as using in vitro and in vivo DNA binding studies.

Figure 1. Model of the various steps in the activation of the chicken lysozyme locus during macro-phage differentiation. (A) In lysozyme non-expressing cells we only find a DNaseI hypersensitive chromatin site (DHS) at the silencer complex located -2.4 kb upstream of the transcription start. (B) In myeloblasts, which represent a cell type still capable of differentiating to granulocytes and macrophages, DHS form at the promoter and at the early (-6.1 kb and -3.9 kb) enhancers. The presence of promoter sequences is essential for DHS formation at the early enhancers. The early enhancers and the promoter are sufficient to activate the lysozyme locus at the correct developmental stage. (C) In terminally differentiated and activated macrophages the silencer complex is inactivated and the third enhancer becomes active leading to a 20-fold increase in mRNA levels. The position of the different cis-regulatory elements are indicated by black bars, the coding region with the first two exons is depicted by striped boxes, and the various cis-regulatory protein complexes are shown as differently patterned shapes. The transcriptional activity of the lysozyme gene is indicated by arrows of different sizes. Note that enhancer complexes are extending over larger regions than the actual core sequences.

Figure 3 shows what we know about the functional elements of the -6.1 kb enhancer and the promoter based on those experiments. In our initial promoterless construct we had deleted sequences from -1 bp to -830 bp. The promoter is a complex element, since it contains three start sites for the RNA polymerase as well as a large collection of ubiquitous and cell-specific transcription factor binding sites. Transcription factor composition of the promoter in oviduct and macrophages is different, which is reflected in a difference of DHS fine structure in the two tissues (Sippel et al., 1988). In macrophages, an additional pair of DHS at -0.7 kb is formed.

However, promoter activity seems to entirely reside on a fragment of approximately 250 bp in length; further upstream, an element has been identified which exerts a negative effect on reporter gene expression in transient

transfection assays in macrophage cell lines (Steiner et al., 1987). Consequently, only the proteins binding to this fragment have been identified as being necessary for promoter function (Altschmied et al., 1989; Dölle and Strätling, 1990) (**Figure 3A**). Using a similar strategy, the functional elements of the -6.1 kb enhancer have been assigned to reside on a sequence array of approximately 200 bp within a 596 bp BamHI - HindIII fragment encompassing the DHS at this position (**Figure 3B**). The minimal enhancer fragment showing full enhancer activity in transient transfection assays was assigned to an even smaller fragment (Grewal et al., 1992). In neither case was it known whether the minimal elements, as defined by transient transfection assays, are sufficient for their correct activation during ontogeny.

Figure 2. Chromatin structure of the wt and promoter-less chicken lysozyme constructs in transgenic mice. Summary of chromatin structure analyses of different constructs in different cell types of transgenic mice as indicated on the left. At the top of each panel the 5'-region of the chicken lysozyme constructs is depicted. The promoter deletion is indicated as a black triangle. The main transcription start is indicated by a horizontal arrow. Exons 1 and 2 are symbolized by grey boxes. Black arrows: DHS displayed at wild type strength irrespective of the chromosomal location of the transgene. Grey arrows: -2.4 kb- and -2.7 kb DHS displaying changes in intensity according to the developmental stage of the cells. Striped arrows: DHS forming with variable efficiency depending on the chromosomal position of the transgene. (A): construct carrying the full set of cis-regulatory elements; (B): construct carrying a promoter deletion.

B. Deletion of flanking sequences abolishes DHS formation at the -6.1 kb enhancer

Our original deletion of the -6.1 kb enhancer which abolished the early onset of transcriptional upregulation had removed a large fragment encompassing sequences from -5400 bp up to -8700 bp. We were now interested to examine whether the 596 bp BamHI - HindIII fragment carried all the information for its interaction with the promoter and, hence, for its chromatin remodelling activity. We generated two transgenic mouse lines which carried a deletion construct as described above but where the 596 bp fragment carrying all the transcription factor binding sites found to be necessary for enhancer activity had been reinserted, thus removing large flanking sequence arrays and placing the -6.1 kb core enhancer element into a new sequence context (**Figure 5 B**). The deletion also removed a non-tissue specific weak DHS at -7.9 kb (see **Figure 5** lane 15) of no apparent cis-regulatory function. As expected, the deletion did not affect the tissue specificity of expression.

As in the transgenic mouse lines carrying the complete locus, the lysozyme gene is expressed in a tissue specific fashion (**Figure 4**). Expression is only observed in hematopoietic tissues and, in case of the BH 596.3 mouse

line, also in the lung, a tissue which can contain a large number of macrophage cells.

The wild type lysozyme locus construct encompassing the complete chromatin domain is expressed also in the brain (Bonifer et al., 1990; Bonifer et al., 1994b). Expression is truly ectopic, but cell-specific and copy number-dependent, indicating that the trans-species transgene is activated correctly in a brain cell type fortuitously containing the correct transcription factor combination (Bonifer et al., 1994a). Brain-specific expression is lost when the -6.1 kb enhancer is deleted (Bonifer et al., 1994b). Interestingly, as opposed to the wild type locus, no ectopic expression of the transgene in the brain is observed with the deletion construct described here, indicating, that the -6.1 kb enhancer is not active in the brain of these mice.

Structural studies confirmed these findings. We isolated macrophages from transgenic mice carrying either the full set of cis-regulatory elements (XS.0b mice) or the above described deletion construct (BH596.1 and BH596.3 mice) and analyzed the DHS pattern in the -6.1 kb- and the -3.9 kb enhancer region (**Figure 4**). We also examined the nucleosomal pattern around the -2.4 kb silencer/ -2.7 kb enhancer region using MNase digestion of nuclei of lysozyme non-expressing embryonic fibroblasts and macrophages of XS.0b and BH 596 mice.

Figure 3. DNA - Protein interactions at the Promoter (A) and at the early -6.1 kb enhancer (B). Summary of in vitro - and in vivo DNA protein interactions at the promoter (A) and at the -6.1 kb enhancer (B) of the chicken lysozyme locus. Transcription factor binding sites as are indicated as differently patterned boxes, the nature of the presumed transcription factor binding to this sequence is indicated above the sequence. The data represent in (A) a compilation of the data from the following references: (Altschmied et al., 1989; Döller and Strätling, 1990; Grez et al., 1981). LS defines linker scan mutations leading to a inhibition of promoter activity as assayed by (Luckow and Schutz, 1989). The arrows in (B) indicate the position of point mutations leading to a loss in enhancer activity as described in (Grewal et al., 1992). The data in (B) represent a compilation of the following references: (Grewal et al., 1987a; Borgmeyer et al., 1984; Sippel et al., 1987b).

Figure 4. Tissue specific expression pattern of BH596 mice. mRNA expression analysis of BH596 mouse lines. Expression of the chicken lysozyme gene in different tissues of two independently derived BH596 transgenic mouse lines. Total RNA (20 μ g) was analyzed in an S1 protection assay with probes specific for chicken lysozyme (**upper panel**) or mouse β -actin (**lower panel**) as described (Bonifer et al., 1990). Abbreviations below the lanes indicate the investigated tissues / cell types. L: liver; H: heart; K: kidney; Lg: lung; S: spleen; B: bone marrow; T: thymus; M: peritoneal macrophage. Lane (-): no RNA; HD11: RNA prepared from HD11 cells stimulated with LPS. The numbers at the right indicate the positions of the three major start sites at the lysozyme promoter (Grez et al., 1981).

Embryonic fibroblasts show a regular MNase pattern indicative of a phased nucleosome over both elements which is perturbed after the activation of the -2.7 kb enhancer in macrophages (**Figure 6**). This pattern is identical in both mouse lines indicating that the deletion does not affect the nucleosomal organization of these elements. In addition, no difference between the mouse lines was observed with respect to the formation of the DHS at the -3.9 kb enhancer. We have already shown that the presence or absence of the -6.1 kb enhancer has no effect on the formation of an active promoter structure (Huber et al., 1996). However, the deletion of flanking sequences strongly affected DHS formation at the -6.1 kb enhancer. In contrast to the mice carrying the complete lysozyme locus which show a strong DHS at the position of the -6.1 kb enhancer, no DHS is formed at the position of reinserted BamHI – Hind III fragment. This indicates that flanking regions are required for DHS formation at the -6.1 kb enhancer. The DNA region required to stabilize a functional enhancer complex at -6.1 kb is obviously much larger than previously anticipated.

At present we do not fully understand the molecular basis of our finding. Several explanations are possible which most likely are not mutually exclusive. Firstly, we know that in its inactive state the -6.1 kb enhancer is organized in a phased nucleosome which is remodelled by

enhancer activation (Huber et al., 1996). It is therefore possible that this preset chromatin structure is disturbed in case of the enhancer deletion, which would in turn lead to a disturbance of enhancer activation. One of the crucial transcription factors involved in enhancer activity is nuclear factor I (NFI) which by itself is unable to bind to DNA organized in a nucleosome irrespective of nucleosome positioning (Pina et al., 1990; Blomquist et al., 1996a). In vivo, this factor requires assistance to be able to bind to its recognition sequence in chromatin (Perlmann and Wrangle, 1988; Truss et al., 1995). In our case, this assistance might be prohibited by a change in chromatin architecture.

Secondly, it is possible that earlier in vivo and in vitro DNA-binding studies have failed to detect low affinity binding sites for factors binding to flanking sequences recruited by the factors binding to the enhancer cores. In vivo a large complex might be formed which is too fragile to be reconstituted in vitro using conventional extract preparation and assembly technology.

Thirdly, the constitutive hypersensitive site at -7.9 kb may be involved in stabilizing the -6.1 kb enhancer complex.

Figure 5. The deletion of flanking sequences around the core of the -6.1 kb enhancer abolishes DNaseI hypersensitive site formation at the -6.1 kb enhancer but not at the -3.9 kb enhancer. (A) DHS mapping with macrophages of mouse line XS.0b (lanes 2 - 5) which express the lysozyme gene in a position independent manner and mouse lines BH596.3 and BH596.1 (lanes 5 - 9 and lanes 10 - 13, respectively). Lane 15: Nuclei prepared from chicken HD11 promacrophage cells. M: size marker. Genomic DNA was prepared, restricted with SphI, transferred to a nylon membrane and hybridized with probe DS indicated at the map at the right. The map on the right indicates the position of SphI restriction sites and the position of the -6.1 kb enhancer DHS in the wt- (grey circle) and the deletion construct (white circle). Note that in the BH596 construct one SphI site is deleted. The position of the -3.9 kb DHS is indicated by a grey oval bar. (B) Map of the wild type locus (upper panel) and the BH596 deletion construct (lower panel). The coding region is indicated by the white box with the exon sequences drawn as black bars and the transcriptional start site as horizontal arrow. The positions of the DHSs mapped in macrophages are shown as vertical arrows, constitutive DHSs are indicated as smaller arrows. The position of the upstream enhancer region and the medial enhancer region are indicated as stippled boxes. The nature of the cis regulatory elements and their position relative to the transcriptional start site are shown in the lowest panel. E: enhancer element; S: silencer element; P: promoter elements. The position and nature of the transcription factors binding to the 596 bp BamHI - HindIII fragment are indicated below the line.

Figure 6. The deletion of upstream sequences does not affect nucleosomal remodelling at the -2.7 kb enhancer. MNase analysis of the -2.4 kb silencer/-2.7 kb enhancer region in the BH596.3 mouse line. Lanes 1 - 6: MNase digestion pattern of nuclei prepared from mouse line XS.0b (restricted with SphI - SacI); lanes 1-3: analysis of MNase digestion pattern in the chromatin of transgenic mouse macrophages; lanes 4 - 6: embryonic fibroblasts. Lane 7: DHS pattern of HD11 nuclei in the analyzed region (symbolized by small grey circles). Lanes 8 - 15: MNase analysis of nuclei prepared from macrophages (lanes 8 - 11) or embryonic fibroblasts (lane 12 - 15) of mouse line BH596.3. The probe used for indirect end-labelling (SpS) is indicated by a stippled box on the map depicted at the right. Prominent MNase cleavage sites are indicated by arrows, cleavage sites only observed in macrophages are indicated by striped arrows. M: Size markers.

Finally, it is possible that the deletion of sequences upstream of the -6.1 kb have brought this element too close to the boundaries of the DNaseI sensitive domain. It has been demonstrated that these regions have insulator properties (Stief et al., 1989). Recently, it could be shown that they contain high affinity binding sites for the chicken homologue of the methyl-binding-protein MeCP2, which acts as a transcriptional repressor (Weitzel et al., 1997). The repressor activity of this protein can be explained by the finding that it is capable of recruiting a histone deacetylase. It is therefore tempting to speculate that the DNaseI-resistant chromatin of the domain borders has spread into the newly inserted -6.1 kb enhancer sequences thus rendering this element inactive. Taken together, these explanations suggest that with our present technology in identifying cis-regulatory elements we are missing out essential regulatory features of eukaryotic gene loci which are crucial for locus activation in a developmentally-regulated system. It also demonstrates that it is important to analyse the composition of cis-regulatory elements in their natural sequence context.

C. All sequences of a eukaryotic locus are part of a functional unit

The activation of the chicken lysozyme locus during cell differentiation is a stepwise process. Our analysis of the developmental activation of the lysozyme locus has demonstrated a definitive requirement for all cis-regulatory elements of the gene locus to cooperate. 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. The differentiation-dependent reorganization and activation of the lysozyme locus is mediated by the interplay of separate cis-regulatory elements with distinct abilities to generate or maintain transcription competent chromatin structures. Our results 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 in transgenic mice.

In the study described here, we demonstrate in addition that the characterization of the early -6.1 kb enhancer by transient transfection assays has been incomplete and that not only a promoter but also enhancer flanking sequences

are required for the developmentally-controlled chromatin remodelling activity of this element. Several laboratories have independently detected a cis-regulatory role for enhancer core flanking regions. The results of our experiments are reminiscent of a study in which the activity of one of the major control elements of the human adenosine deaminase (ADA) gene was examined. This control region is located in the first intron and is essential for the correct activation of the ADA locus in transgenic mice (Aronow et al., 1995). It has also been demonstrated in this system that a core enhancer region is insufficient for the activation of the enhancer in transgenic mice. Flanking regions are required which have no enhancer activity on their own.

In another study the role of sequences outside the cores of the DNaseI hypersensitive sites of the β -globin LCR has been examined (Jackson et al., 1996). Also here, a cis-regulatory role of these regions could be established. It was found that a strong synergism in transcriptional stimulation was observed when sequences outside the cores were present and the natural spacing between the hypersensitive sites was preserved. A sequence comparison between β -globin LCRs of different mammals indeed revealed a high level of sequence conservation of certain core flanking sequences (Slightom et al., 1997), indicating an important role of these sequences in LCR function. At present it is unknown which type of information is encoded in enhancer core flanking regions. The fact that individual sequence motifs are evolutionary conserved, however, points to the presence of important transcription factor binding sites. It may also be possible that structural information is present which, at the moment, we are unable to identify.

What we can detect with our classical techniques like DNaseI hypersensitive site mapping, in vitro DNA binding studies and transient transfection studies may be nothing but the "tip of the iceberg" in terms of clusters of high affinity transcription factor binding sites. We might be unable to detect scattered single or low affinity factor binding sites. Taken together it is obvious that gene locus activation is a cooperative process. What emerges from our work and the above described experiments is the concept that this cooperative process may involve all sequences of a eukaryotic gene locus, some of which span hundreds of kilobases. The elucidation of the type of information encoded in these sequences and the way this information is translated into the enormous complexities of developmentally-controlled gene expression will be a major challenge for developmental biologists in the next years.

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VI. Materials and Methods

A. Construction of pIIIlysBH596

The pIIIlysBH596 plasmid was constructed by cloning the BamHI – HindIII fragment carrying the -6.1 kb enhancer (Theisen et al., 1986) in sense orientation into the Asp718 site (at -5.4 kb upstream of the transcription start) of plasmid PIIIlysdXK (Bonifer et al., 1994b) which carried a deletion of a fragment between -5.4 kb and -8.7 kb.

B. Transgenic mice, cell culture and mRNA expression analysis

Production of the BH596 transgenic mouse lines by pronuclear injection of DNA was essentially performed as described in Hogan et al. (1994). First-generation heterozygous mice from the founders BH959.1 and BH593.3 were examined for intact integration and construct integrity by Southern blotting. Copy-numbers were calculated from Southern blots as described by Bonifer et al. (1990) and phosphorimager analysis. BH 596.1 mice carried 2 copies and BH596.3 mice carried 18 copies of the lysozyme locus. Expression and chromatin analysis were performed with homozygous progeny. Transgenic mouse lines carrying the XS construct (Bonifer et al., 1994b) were kept as homozygous lines in our mouse colony. Primary macrophages were prepared from the peritoneal cavity of transgenic mice as described (Bonifer et al., 1990). 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 hours. Embryonic fibroblasts were prepared from mouse embryos 12 days after fertilization as described earlier (Huber et al., 1994). HD11 cells were grown in standard Iscove's medium containing 8% FCS and 2% chicken serum. Preparation of mRNA and the S1 protection analyses were performed as described in (Bonifer et al., 1990).

C. Nuclei preparation

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

D. DNaseI and MNase digestion analysis

Aliquots of 2×10^7 to 1×10^8 nuclei in 100-200 μ l of buffer 3 were centrifuged for 5 min at 600g and 4°C and thereafter resuspended in buffer 4 (0.15mM spermine, 0.5mM spermidine, 15mM Tris-HCl pH 7.5, 60mM KCl, 15mM NaCl, 0.2mM EDTA, 0.2mM EGTA). DNaseI digestions were performed in 500 μ l buffer 4. To 2×10^7 nuclei 0, 4, 10, 20 and 40 Units/ml DNaseI (Pharmacia) were added. HD11 nuclei were digested with 24 Units/ml DNaseI. Digestion was started by adding 4mM

MgCl₂ and 2mM CaCl₂. Incubations (15 min, 4°C) were stopped by adding 10μl 0.5M EDTA. MNaseI digestions were performed in 200μl buffer 4. To 2x10⁷ nuclei 0, 15, 80 Units MNase (Pharmacia) were added. Digestion was started by adding 10μl CaCl₂ (100mM) and stopped after incubation (5 min, 25°C) by the addition of 10μl 0.5M EDTA.

Digestion of naked genomic DNA with MNase was performed in 150μl 10mM Tris-HCl pH7.5 with 0.2 - 6.4 Units/ml MNase. Incubations (15 min, 25°C) were started by adding 15μl CaCl₂ (10mM) and stopped with 15μl 50mM EDTA. After DNaseI or MNase digestion nuclei were lysed in 500μl Tris-HCl pH 8.0, 2mM EDTA, 0.2% SDS, 0.5mg/ml Proteinase K and incubated overnight at 37°C. RNase A (0.2mg/ml) was then added and after a further incubation at 37°C for 1h the DNA was precipitated three times with ethanol. Digested DNA was cleaved with restriction enzymes for indirect end-labelling analysis and 7 - 30μg of fragmented DNA were loaded on 3mm thick vertical 1% agarose gels (DNaseI analysis) or 10mm 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 (a SphI-SpeI fragment from -3163 to -2906bp) for indirect endlabelling.

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