Minireview

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The Chicken Lysozyme Locus as a Paradigm for the Complex Developmental Regulation of Eukaryotic Gene Loci*

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Gene loci of higher organisms have complex structural features. In some cases their coding regions occupy many, even hundreds, of kilobases of DNA. Additionally, the sequences that contain the information for the correct spatial and temporal regulation of a particular gene locus during development often exceed the extensions of the coding region by severalfold. The question of what type of information is encoded in these vast amounts of DNA has puzzled researchers from the beginning.

It is now clear that eukaryotic genes are regulated by a number of different cis-regulatory elements distributed over large distances. A convenient way to assay the number and the distribution of cis-regulatory elements has been the mapping of DHS[1] in chromatin. Such local chromatin perturbations are in most cases caused by the binding of transcription factors to their cognate DNA sequences. The pattern of DHS can undergo dramatic developmental changes, indicating a change in the activity of cis-regulatory elements. In addition, the analysis of protein-DNA interactions at a single-nucleotide resolution level *in vivo* has demonstrated that, depending on the developmental stage, different combinations of transcription factors can occupy the same cis-regulatory element (1, 2). These experiments indicate that the transcriptional activation of a gene locus is achieved by the cooperation of several different cis-regulatory elements, which, in turn, assemble transcription factors in a sequential, developmentally controlled fashion. However, the assembly of active transcription factor complexes on natural genes does not occur on naked DNA but in a chromatin context, where nucleosome-DNA interactions have to be counteracted. Hence, the activation of a gene locus requires at least the following steps: the perturbation of chromatin structure by the binding of transcription factors on cis-regulatory elements, the developmentally controlled reorganization of transcription factor complexes, the assembly of the basal transcription machinery and its interaction with upstream regulatory elements, the onset of mRNA synthesis, and, in many cases, the maintenance of an active transcriptional state during multiple rounds of DNA synthesis.

How can the molecular basis of locus activation be experimentally studied? While the basal activities of individual cis-regulatory elements of particular gene loci can be analyzed by transient and stable transfection experiments, the molecular mechanism of activation of a gene locus from the transcriptionally silent state can only be studied in a developing system, preferentially in transgenic animals. The ideal model locus should be small, thus facilitating the manipulation of individual cis-regulatory elements within the context of an entire genomic locus, and it should be extensively characterized on the molecular level. In addition, to dissect the role of different cis-regulatory elements in the developmental control of gene locus activation, it should be possible to follow cell differentiation experimentally, thus enabling the linkage of a stage-specific chromatin structure with the transcriptional activity of the gene. Here, we summarize recent studies on the molecular basis of the transcriptional activation of the chicken lysozyme locus, which may serve as a paradigm for other developmentally regulated eukaryotic gene loci.

The Complete Chicken Lysozyme Locus Comprises the Regulatory Unit of Transcription

The chicken lysozyme gene is expressed in the mature oviduct and in cells of the myeloid lineage of the hematopoietic system (3, 4). In myeloid cells the gene is up-regulated during the differentiation of multipotent myeloid progenitor cells to mature granulocytes and macrophages (5, 6). The structural dimensions of the chicken lysozyme locus are defined by an increased general DNase I sensitivity of chromatin over an array of 24 kb around the transcribed region (7). All DHS and thus also all cis-regulatory elements are confined within this chromatin domain (5, 6, 8, 9). Transfection analysis revealed three enhancers (at −6.1 kb, −3.9 kb, and −2.7 kb), a hormone-responsive element (−1.9 kb), a silencer element (−2.4 kb), and a complex promoter (see Refs. 10 and 11 and Fig. 1). The borders of the DNase I-sensitive domain coincide with sequences binding to the nuclear matrix *in vitro* (12), suggesting that the lysozyme locus forms a chromosomal loop (13). However, at present it is unclear whether these sequences are firmly attached to the nuclear matrix *in vivo* (14).

Experiments in transgenic mice demonstrated that the complete, structurally defined lysozyme gene locus is specifically expressed in the right cell type (macrophages) and is unaffected by chromosomal position effects (15). The individual contributions of the different cis-regulatory elements to differentiation-dependent transcriptional activation of the entire gene locus were revealed by analyzing deletion mutants (16). Each construct with a deletion of one enhancer region supports macrophage-specific expression. However, position independence of expression is lost as soon as one essential cis-regulatory region is deleted. Thus, for correct locus activation in development the cooperative action of all cis-regulatory elements is necessary. At present, the role of the domain border fragments remains elusive, since their deletion does not abolish copy-number dependence of expression. Only if one enhancer region in addition is deleted is a substantially higher incidence of ectopic expression observed as compared with constructs with domain border fragments, together with the abrogation of position independ-

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[1] The abbreviations used are: DHS, DNase I-hypersensitive chromatin site(s); kb, kilobase(s); LCR, locus control region; MNase, micrococcal nuclease.

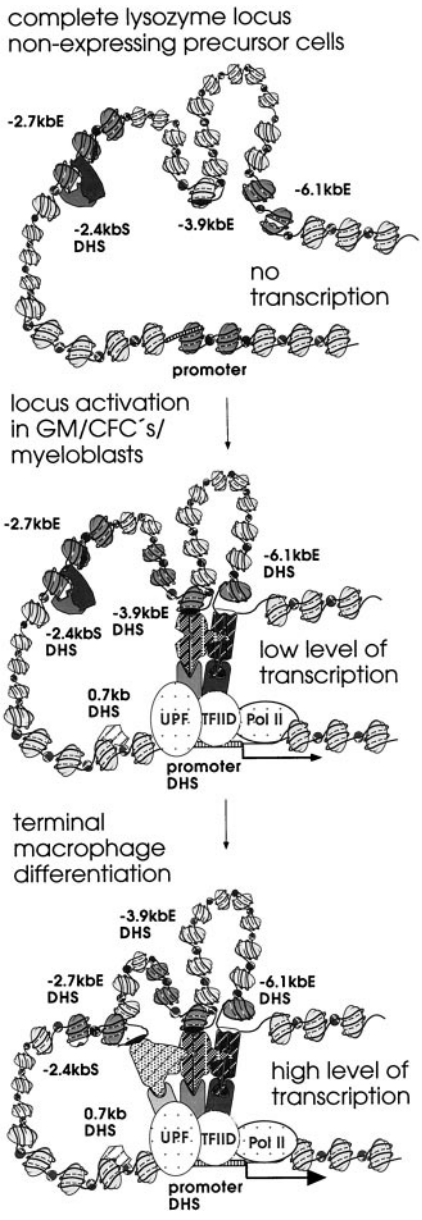

FIG. 1. **A model for the developmental reorganization of chicken lysozyme chromatin.** Chromatin structure of the chicken lysozyme locus 5′-regulatory region in different cell types is shown. The positions of cis-regulatory elements as well as their nature are indicated. Hypersensitive DNase I cleavage sites are indicated as nonhistone proteins (various shapes) and by the indication *DHS*. Phased nucleosomes determined by MNase digestion analysis and indicated by a regular 150–200-bp distance of MNase cuts are depicted in *dark gray*; *light gray* nucleosomes indicate areas with no prominent MNase cleavage sites. *UPF*, upstream promoter factors binding to a stimulatory element around 200 bp upstream of the transcriptional start site; *TFIID* and *Pol II*, basal transcription machinery; *E*, enhancer; *S*, silencer; *GM/CFC*, granulocyte/macrophage colony-forming units.

ence. The notion that a complete gene locus is resistant against genomic position effects has been used to correctly express transgenes for which the structural and functional extensions were not known, by introducing large yeast artificial chromosomes into the germ line of mice (17, 18).

The Role of Chromatin Architecture in Gene Regulation

The question now emerges as to whether a gene locus is completely defined as a collection of cis-regulatory elements, which, in turn, are defined by clusters of trans-factor binding sites separated by inert DNA sequences or whether there is more to it than that. How do the coordinated interactions between the cis-regulatory elements take place and which role does chromatin structure play in this process? By analyzing the chromatin structure of the lysozyme locus in chicken macrophage cell lines, we determined the structural reorganization of the various cis-regulatory elements at different developmental stages (6). It turns out that the chromatin of the lysozyme locus is reorganized in several steps, depending on the differentiation stage of the cells (Fig. 1). The lysozyme gene in multipotent progenitor cell lines is transcriptionally inactive and exhibits the chromatin configuration characteristic for lysozyme nonexpressing cells. Only the DHS at the −2.4-kb silencer is present. At the myeloblast stage DHS appear at the −6.1-kb enhancer, the −3.9-kb enhancer, and at the promoter. At subsequent differentiation stages, the DHS at −2.4 kb disappears and a DHS at the −2.7-kb enhancer is formed. Transcriptional activity increases from a very low level in myeloblasts to a 100-fold higher activity in bacterial lipopolysaccharide-stimulated, activated macrophages. 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. Subsequent studies used MNase to probe for specifically positioned (phased) nucleosomes in the complete 5′-regulatory region of the lysozyme locus. In the transcriptionally inactive state phased nucleosomes are located at most cis-regulatory elements (19), as schematically depicted in Fig. 1. Each cis-regulatory element shows a unique structural organization, with transcription factor-binding sites specifically arranged with respect to nucleosomes. Transcriptional activation results in significant rearrangements of chromatin structure, which, however, are of different natures in different cis-regulatory elements. At the promoter we see a regularly spaced MNase pattern indicative of the presence of phased nucleosomes. After gene activation we see a perturbation of this pattern. The same is found at the −6.1-kb enhancer. In contrast, as in the case of the mouse albumin enhancer (20), nucleosomes seem to be actively positioned after the activation of the −3.9-kb enhancer. The situation is even more complicated at the −2.4-kb silencer/−2.7-kb enhancer region. Here, nucleosomes seem to be specifically positioned in the presence or absence of a DHS at the silencer element, indicating that transcription factor assembly occurs on their surface, similar to what is observed at the mouse mammary tumor virus promoter (21).

The results of our structural studies suggest that the correct alignment of transcription factor-binding sites with respect to the position of nucleosomes is essential for their undisturbed interaction. Chromatin at the inactive state of the gene seems to be preset and seems to fold the gene locus into a distinct three-dimensional structure, thus leading to a precise spatial alignment of DNA sequences. Based on these observations, we hypothesize that every DNA sequence on a gene locus serves a purpose. Support for this idea comes from experiments in which constructs containing combinations of very short fragments encompassing only the minimal regulatory regions of the lysozyme locus were analyzed in transgenic mice. These constructs were not expressed.[2] One of the reasons for their failure might be that they are unable to support the gradual chromatin rearrangements necessary to activate the gene locus during cell differentiation. In this context it is also interesting to note that deletion of a single DHS of the major upstream control region of the human β-globin locus, the LCR, when analyzed in transgenic mice in the context of the complete

[2] C. Bonifer, M. Vidal, F. Grosveld, and A. E. Sippel, unpublished observations.

β-globin locus, leads to a breakdown of position independence of expression (22). This is in contrast to what is observed with smaller constructs in which sequences between the LCR and the β-globin genes have been deleted. Here, it may be speculated that, in analogy with the tethered loop model of gene silencing put forward by Pirrotta (23), transcription factors binding to high affinity binding sites on core enhancer elements of natural gene loci have to recruit proteins on weaker binding sites on flanking sequences. All elements may be necessary to bridge the large distances to the promoter and to fold a gene locus into the active conformation.

The Role of Positive and Negative Cis-regulatory Elements in Lysozyme Locus Activation

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 phasing pattern as well as for the reorganization of the −2.4-kb/−2.7-kb region after terminal macrophage differentiation (19), demonstrating that the same chromatin rearrangements take place in both species. Our experiments with deletion mutants of the lysozyme locus in transgenic mice demonstrated that each enhancer region is capable of driving expression in mature macrophages (16). We then asked how the different cis-regulatory elements cooperate during earlier stages of cell differentiation. To this end, we 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 (24). In early macrophage precursor cells the −6.1 kb and −3.9-kb enhancers, the promoter, and the silencer element are DNase I-hypersensitive, whereas the −2.7-kb enhancer is not (6, 10, 19). Our experiments demonstrate that a construct carrying only the early (−6.1 kb and −3.9 kb) enhancers is capable of activating the lysozyme locus at the same developmental stage and that transcription is up-regulated with the same kinetics as the wild type locus carrying all cis-regulatory elements. We conclude from these experiments that the early enhancers are responsible for locus activation and that the onset of chromatin rearrangement at those elements is coupled with the onset of mRNA synthesis. In turn, since a deletion of the −2.4-kb silencer/−2.7-kb enhancer region has no influence on the locus activation kinetics, this implies, in addition, that the silencer element does not repress the action of the early (−6.1 kb and −3.9 kb) enhancers. In concordance with the chromatin data, a construct in which the −6.1-kb enhancer has been deleted shows a delay in transcriptional activation.

Our structural analyses might present a clue to the role of the −2.4-kb element. We have demonstrated that the silencer element and the immediately juxtaposed enhancer element are each organized in a positioned nucleosome and most likely form an integrated cis-regulatory element (Fig. 1). The spacing of binding sites is such that they may face the same side on each nucleosome, thus bringing them into close contact (19). The exact position of the nucleosomes is not yet known and awaits the structural analysis of this region at the single-nucleotide resolution level. However, chromatin rearrangements at the −2.4-kb and −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, we regard it as most likely that the −2.4-kb element is repressing the macrophage-specific −2.7-kb enhancer element at early developmental stages of myeloid differentiation.

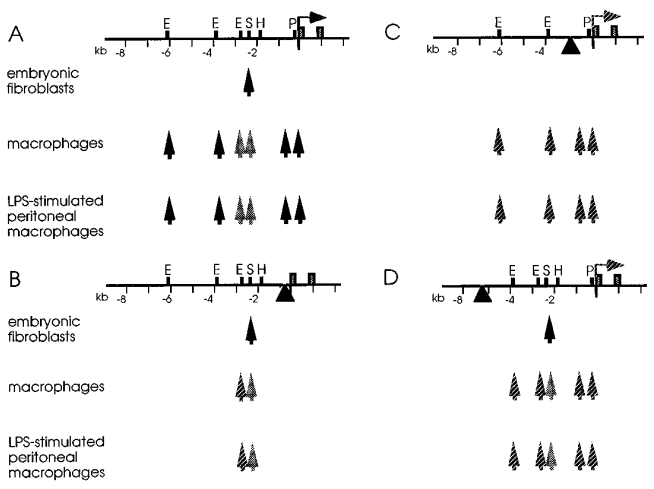

FIG. 2. **Chromatin structure of the various chicken lysozyme constructs in transgenic mice.** A summary of chromatin structure analyses of different constructs in different cell types of transgenic mice is indicated on the *left*. At the *top* of each *panel* the 5′-region of the chicken lysozyme constructs with specific deletions indicated as *black triangles* is depicted. The main transcription start is indicated by a *horizontal arrow: black arrow*, high level transcription; *striped arrow*, variable transcriptional level due to position effects. Exons 1 and 2 are symbolized by *gray boxes*. *Black vertical arrows*, DHS displayed at wild type strength irrespective of the chromosomal location of the transgene; *gray vertical arrows*, −2.4- and −2.7-kb DHS displaying changes in intensity according to the developmental stage of the cells; *striped vertical 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; *C*, construct carrying a deletion of the late enhancer region; *D*, construct carrying a deletion of the early enhancer region. *LPS,* bacterial lipopolysaccharide; *E*, enhancer; *S*, silencer; *H*, hormone-responsive element; *P*, promoter.

What is the role of the promoter in lysozyme locus activation? To answer this question we analyzed a construct carrying the complete lysozyme locus with an internal deletion of the lysozyme promoter in transgenic mice (Fig. 2). Transcription from this construct was completely abolished (25). Surprisingly, the deletion of promoter sequences uncovered a difference in the intrinsic ability of the individual cis-elements of the chicken lysozyme locus to withstand repressing chromatin conformations. The formation of a DHS at the −2.4-kb silencer element was unaffected, and also the DHS at the −2.7-kb enhancer element was formed, albeit with different intensity, depending on the chromosomal position. In contrast, DHS formation at the early −6.1-kb and −3.9-kb enhancers was abolished. Obviously, in the initial activation of the lysozyme locus the early enhancers have to interact with the promoter to form a stable enhancer-promoter complex. Hence, the chicken lysozyme locus harbors no single element with dominant chromatin opening function that is sufficient for locus activation. Although elements (2.7-kb enhancer/2.4-kb silencer) exist that are able to reconfigure the chromatin promoter independently, they act later in cell differentiation, and their chromatin reorganizing capacity is limited to their site.

Taken together our data indicate that initial locus activation is performed by the interaction of the −6.1-kb and −3.9-kb enhancers with the promoter. Maximal transcriptional activity is achieved by the inactivation of the silencer element and the simultaneous activation of the −2.7-kb enhancer (Fig. 1). One question, however, is immediately apparent: if the early enhancers are sufficient to activate the lysozyme locus at the correct developmental stage, why is the complete locus necessary for position-independent expression?

Incomplete Gene Loci Do Not Form Stable Transcription Complexes

When complete gene loci are expressed independent of genomic position, gene expression levels per gene copy are constant, whereas incomplete gene loci express variable levels per gene copy, depending on the site of integration. With deletion constructs expressing at a low expression level per gene copy we could show that the formation of DHS at the enhancers and the promoter is suppressed (26). The degree of suppression of DHS formation is characteristic for each individual mouse line (Fig. 2). In addition, we analyzed by MNase digestion the chromatin of lysozyme transgenes expressed in an integration site-dependent fashion (19). Individual mouse lines carrying the same construct but with different expression levels were compared. Thus, it could be determined whether all transgene copies within a multicopy transgene cluster adopt the same chromatin configuration or whether MNase patterns characteristic for active and inactive loci are superimposed on each other. The conclusion is that chromosomal position effects do not influence nucleosome positioning. Instead, our analysis shows that integration site-dependent expression of transgenes is associated with a mixed MNase pattern, indicating the presence of active and inactive transgenes within the same multicopy cluster. Hence, not all gene copies within a single multicopy transgene cluster are transcriptionally active (at a given time). The proportion of active and inactive gene loci depends on the chromosomal environment and the nature of the introduced mutation. The idea of a dynamic equilibrium between the active and the inactive state of a gene locus, which (in the case of transgenes) is a function of the genomic integration site, is supported by a variety of studies. In a series of elegant experiments, Fraser and co-workers (27) have demonstrated that the human β-globin LCR-promoter interaction is dynamic and switches between several promoters of the downstream located globin genes (25). The authors also analyzed certain transgenic mouse lines carrying deletion mutants of the complete β-globin locus, which render the transgene susceptible to genomic position effects. The same level of steady state mRNA was observed in each cell; however, in contrast to the complete locus, not all cells show primary transcript synthesis at the same time (22), indicating that in each cell the interaction of the LCR with the promoter is unstable. In a different study it was shown that enhancers, when analyzed in stably transfected cells, act as on-off switches, rather than by increasing transcription rates. It was concluded from these experiments that enhancers increase the probability of forming a stable transcription complex at the promoter by antagonizing repressive chromatin structures (28). Translated into a situation where several cis-regulatory elements have to cooperate, this result indicates that the stability of the interaction of transcription factor complexes on a given gene locus is decisive for its sustained activity. In the case of the lysozyme locus this would imply that one enhancer region is sufficient to activate the lysozyme locus; however, to reproducibly maintain transcriptional activity and an active chromatin structure at all chromosomal locations, all cis-regulatory elements are necessary.

Perspectives

Recent experiments studying the molecular basis of gene locus activation in development have uncovered a stunning complexity of regulatory principles. In particular, they point to an important role of chromatin organization in this process. Pattern formation is based on successive phenotypical changes of cells, whereby spatial information, through cell-cell interactions, is often translated into a change in cell identity. On the molecular level, these processes are reflected at the different levels that control gene expression. "Historic" information, that is information as to where cells have been before and with whom they have communicated, is important for correct pattern formation. In the same way, the order of chromatin structure reorganization during the various cellular differentiation states may be important for correct gene locus activation. If this process is disturbed, something goes wrong. This concept will undoubtedly influence the design of experiments examining the molecular basis of gene expression control.

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