CONTROL OF EXPRESSION OF THE CHICKEN LYSOZYME GENE AND ITS POTENTIAL APPLICATION IN TRANSGENIC BIRDS

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SUMMARY

The full complement of cis-active DNA sequences for the control of transcription of the chicken lysozyme gene was determined. The functional unit comprises a 21 kb chromatin domain containing the gene, all regulatory elements and 5' and 3' domain border regions. The lysozyme gene domain DNA is able to mediate consistently correct cell type specific gene expression, is able to overcome genomic position effects and is a potential vector for the production of foreign proteins in the avian egg.

INTRODUCTION

The transfer of genes into the germ line of organisms is an attractive genetic route for new livestock production. However, genes randomly inserted into the host genome are most often incorrectly regulated, are not expressed in the desired tissue or are expressed at low levels. Independently derived transgenic organisms show, depending on the chromosomal site of integration, variable transcriptional efficiencies and different tissue specific expression patterns of the same DNA construct. Position effect is a term used to describe phenomena in which the activity of a gene is affected by its location in the genome (for review see Lima-de-Faria, 1983). Difficulties caused by the genomic position effect can be circumvented by altering gene sequences at their natural location by homologous recombination (Capecchi, 1989). Gene targeting by homologous recombination is, however not applicable when foreign genes are newly introduced into the host genome or when experimental protocols are used in which cellular selection is prevented. For these reasons strategies need to be developed to overcome the unpredictable influence of position effects. By better understanding gene regulation and the mechanisms which cause chromosomal position effects, we expect to be able to develop vector systems which allow random integration of transgenes in such a way that they behave independently of their neighboring host DNA sequences.

Planning to develop vector systems for highly efficient tissue specific expression of commercially interesting proteins in secretory glands of farm animals, we originally cloned cDNAs for mammalian milk proteins (Hennighausen and Sippel 1982) and for avian egg white proteins (Sippel et al. 1978) and their genomic gene sequences. The gene for lysozyme, one of the egg white proteins, is transcriptionally activated by steroid hormones in the tubular gland cells of hen oviducts or during cell maturation in macrophages (Sippel et al. 1988). Tissue specific activation of the gene results from the combined function of several regulatory DNA elements within a chromatin domain of 21kb (Sippel et al. 1988). We show here that the deregulating influence of neighboring chromosomal regions on randomly integrated transgenes is eliminated by the concerted action of the full set of regulatory cis-acting elements and is buffered by domain border regions. Genomic position effects can best be overcome if entire functional domain DNAs (rather than incomplete subdomain DNA fragments) are used as vectors for the stable transfer of genes into the genome of transgenic animals.
MATERIALS AND METHODS

General DNase sensitivity and DNasel hypersensitive sites (DHS) of chromatin. Isolated nuclei were digested with DNasel, genomic DNA was purified and analyzed as described in Jantzen et al., 1986 (first cut method) and in Fritton et al., 1984 (DHS mapping).

Transient transfection assays to test the functional specificity of cis regulatory DNA elements. Respective reporter gene constructs were transferred into various cells in culture and analyzed as described in Grewal et al., 1992.

Stable transfection assays to test the function of DBRs (A-elements). Construction and analysis of cell clones, representing individual genomic integration events, were performed as described in Stief et al., 1989. Site specific integration experiments using the S.cerevisiae FLP recombinase system were carried out according to the protocol of the supplier (Stratagene).

Chicken lysozyme transgenic mice. Generation and characterization of transgenic mice was described in Bonifer et al., 1990.

RESULTS

The chromatin domain of the chicken lysozyme gene

For theoretical reasons it was predicted that in order to permit differential transcriptional activity chromosomal DNA must be topographically partitioned into independent functional units (Jackson, 1986). To prove the existence of regulatory units for transcription along the DNA in vertebrate nuclei we have studied the chromatin structure of a specific model gene, the gene for chicken lysozyme. When transcriptionally active in steroid stimulated tubular gland cells of the hen oviduct or in late stage cells of the myelo-monocytic lineage of the hematopoietic system the gene is located within a chromatin domain characterized by high general DNase sensitivity of its DNA (Sippel et al. 1993). The region of

Figure 1. The active chromatin domain of the chicken lysozyme gene.
A. The relative DNasel sensitivity of DNA in chromatin of isolated nuclei of laying hen oviduct cells and chicken premacrophages is shown over 40kb of genomic DNA containing the lysozyme gene (exons black boxes, introns open boxes). Arrow heads point to the positions of regulatory elements with numbers giving their location in kb in respect to the transcriptional start site (arrow). -6.1E early myeloid and oviduct cell enhancer (Grewal et al. 1992), -2.7E late myeloid enhancer (Müller et al. 1990, Müller 1990), -2.4S silencer (Baniahmad et al. 1990), -1.9H hormone responsible element in oviduct cells (Hecht et al. 1988, Hecht 1990) and -0.1P lysozyme promoter. Hatched boxes show the positions of MAR sequences colocating the chromatin domain border regions (5' and 3' DBR).
B. The 21.2kb wild type lysozyme gene domain DNA includes the two DBRs, the distal (dis.) and the medial (med.) enhancer region, the promoter and the transcribed gene region.
"open" chromatin extends in both directions far beyond the transcribed region of the gene. Fig. 1A shows that the transition regions from open to closed chromatin in both directions were mapped to the same DNA sequences in oviduct and premacrophage cells. The two domain border regions co-located with previously mapped MAR sequences (Phi-Van and Strätling, 1988), indicating that the chromatin domain of the gene may in both cell types be confined in a chromosomal loop by nuclear matrix attached border regions.

In addition, maximally nine DNase hypersensitive sites (DHSs) have been mapped in the chromatin of the chicken lysozyme gene region from -20kb to +20kb in various cell types (Fritton et al. 1984, Sippel et al. 1988). All of them are located within the domain of generally DNase sensitive chromatin (Fig. 1A). Chromatin DHS mark the positions of cis-active regulatory protein-DNA complexes. Their clustering around the gene within the boundary regions of the chromatin domain strongly suggests that they are all part of a functional unit responsible for the transcriptional control of the gene.

Cell-type and cell-stage specificity of the regulatory elements

A strong argument for a complex mechanism of multielement regulation of transcription was derived from the functional specificities of five tested cis-active elements located in the 5′ flanking part of the gene domain. Figure 1 shows the position of the promoter, various enhancers and a silencer element as identified by transient transfection assays performed in myeloid cells of different developmental state and of various other hematopoietic and non-hematopoietic chicken cell types in culture (Sippel and Renkawitz, 1989). We found two types of results: (1) the presence of a cell-type specific DHS in the chromatin of the endogenous gene locus of a certain cell type correlates with the cis-regulatory activity of the DNA element in the functional cell transfection assay (Sippel et al. 1992). (2) as deduced from the functional specificities, each individual regulatory element turned out to be responsible for a certain sub-aspect of the transcriptional control of the gene. Only the combined action of all of the regulatory elements is able to explain the global control of the chicken lysozyme gene in oviduct and hematopoietic cells.

Position effect on transgene activity in cells in culture

Whereas the regulatory specificities of the DHS elements could be determined in transient DNA transfection assays, the function of DNA from domain border regions (DBRs) of the gene domain could only be detected after stable random genome reinsertion of reporter gene constructs (Stief et al. 1989). The activity of transgene constructs is normally subject to a pronounced position effect, leading to different (mostly repressed) transcriptional efficiencies depending on the genomic site of integration. An example for this is shown in Fig. 2A. The activity of the reporter gene construct varies within 2-3 orders of magnitude between cell clones representing individual genomic insertion events. The pronounced differences in gene activity are due to the influence of the various random neighboring DNA sequences as is demonstrated by our results shown in Fig. 2B. We find no position effect when independent cell clones are compared, which all carry the reporter gene at the same genomic site after sequence specific integration. In a clonal position effect assay we previously have shown that the 5′-DBR (or A-element for attachment element) of the chicken lysozyme gene had a position effect buffering activity when two copies framed reporter gene constructs (Stief et al. 1989). Fig. 2A demonstrates that the position effect buffering activity of the 5′-DBR cannot only be detected in chicken macrophages but also in non-homologous mouse fibroblasts.
Figure 2. Position effect on reporter gene constructs in cells in culture.
A. Mouse fibroblasts (Ltk') were co-transfected with a neomycin resistance gene and the reporter gene construct containing the BK-virus enhancer (E<sup>6</sup>X), the lysozyme promoter (P), and the CAT-gene (C) or the same construct framed by the 5' DBR (A for attachment element) of the chicken lysozyme gene. Individual neomycin resistant cell clones where grown, characterized for integrity and copy number of inserted reporter gene DNA and assayed for relative chloramphenicol acetyl transferase activity.
B. African green monkey kidney CV-1 cells containing a FRTB-gal cassette for site specific recombination with FLP yeast recombinase (Stratagene CV1 clone E25B2) were co-transfected with the FLP expression plasmid (OG44) and plasmid DNA containing the neomycin resistance gene, a FRT site and the reporter gene construct containing the SV40 enhancer-promoter region (E<sup>SV</sup>Psv) and the luciferase gene or the same reporter gene construct framed by the chicken lysozyme 5-DBR (A). White Bgal minus cell clones were grown, characterized as described in A and assayed for relative luciferase activity.

Table 1.
Expression of wild type and deletion mutants of the chicken lysozyme gene domain in transgenic mice

<table>
<thead>
<tr>
<th>transgene construct&lt;sup&gt;a&lt;/sup&gt;</th>
<th>total No.</th>
<th>cell type specific expression&lt;sup&gt;b&lt;/sup&gt;</th>
<th>expression in macrophages</th>
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<tr>
<td></td>
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<td>(No. of strains)</td>
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<tr>
<td>wt</td>
<td>7</td>
<td>7</td>
<td>high</td>
</tr>
<tr>
<td>Ad</td>
<td>5</td>
<td>3</td>
<td>v.l.&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Am</td>
<td>7</td>
<td>7</td>
<td>v.l.</td>
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<tr>
<td>A</td>
<td>5</td>
<td>5</td>
<td>high</td>
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<tr>
<td>Ad,A</td>
<td>12</td>
<td>0</td>
<td>v.l.</td>
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<tr>
<td>Ad,A,m</td>
<td>4</td>
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<td>v.l.</td>
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a Transgene constructs contained either the complete gene domain as shown in Fig. 1B (wt) or constructs in which the distal enhancer region (△d), the medial enhancer region (△m) or/and the DBRs (△A) are deleted.

b Wild type expression pattern is defined by a high expression pattern in macrophages and ectopic expression is defined by expression in non-typical cell types.

c v.l. denotes variable low expression levels from strain to strain.
The locus control function of the gene in transgenic mice

It is conceptually difficult to test for cell type specific transgene expression in cultured cells. Transgenic animal systems must be applied to determine the true cis-active DNA responsible for correct transcriptional regulation of a transgene. We therefore assayed the DNA of the complete wild type chicken lysozyme gene domain plus various deletion mutants in transgenic mice. We wanted to map the necessary parts of the locus which mediate tissue specific correct transgene activity independent of its position of genomic integration. Table 1 shows the result of an experiment in which 40 independent transgenic mouse strains were constructed and tested for their transgene expression pattern. We find that the complete wt domain DNA consistently mediates the correct cell-type specificity and a high level of expression and thus by definition contains the locus control function (Bonifer et al. 1990). Any deletions tested either increase ectopic expression of the transgene or lead to variably low expression with the same DNA construct in different mouse strains or both. Deletion of the DBRs leads to an increase in ectopic expression in non-typical cell types. Both enhancer regions are individually able to mediate macrophage specific expression. However, as soon as one of the cis-regulatory regions is deleted the position independency of expression and thus the locus control function is lost. Consistently high and copy number dependent expression is found only when all regulatory parts of the gene domain are present.

Figure 3. The concerted action model for eukaryotic gene regulation.

A. The multifactorial promoter protein complex is saturated by direct interactions with two multifactorial enhancer protein complexes (E1, E2; thin lines with arrowheads denote DNA from 5' to 3' direction).
B. The non-saturated promoter-enhancer complex interacts non-specifically with an enhancer complex (Ex) in neighboring DNA (---); two triangles depict the position of the gene domain DNA deletion.

DISCUSSION

Previous studies of chromatin structure and of transfected DNA function in cells in culture suggested that all cis regulatory sequences of the chicken lysozyme gene are contained in a chromatin domain located between the 5' to the 3' border regions. Final proof for this assumption comes from the transcriptional analysis of the chicken lysozyme gene locus in transgenic mice. The domain DNA consistently transmits correct cell specific expression in macrophages and behaves as a regulatory unit independent of its random position in the host genome. As soon as one of the enhancer regions is deleted, the locus control function is lost, indicating that locus control is not a feature of a single cis active site
in the gene locus. The complete regulatory part of the wild-type gene domain with or without DBRs turns out to be resistant to genomic position effects. The most likely interpretation of this finding is schematically shown in Fig. 3. In case all regulatory protein-DNA complexes in the locus are able to interact with the preinitiation complex at the promoter, deregulating interactions with random neighboring regulatory complexes appear to be blocked. If however, the concerted action of all regulatory elements is not possible, due to deletion of one or more of them, a non-saturating situation is created for the large regulatory promoter complex and it may become subject to positional influence. In the latter case deregulatory effects appear to become more difficult by the presence of insulating domain border sequences. The DBRs significantly reduce ectopic expression of incomplete regulatory units. However, they are not (as) necessary for position effect resistance when the complete set of regulatory elements of a gene is transferred to the host genome. These results from clonal position effect assays imply, that it is possible to design transgene vectors for the predictable and correct expression of randomly inserted transgenes after their complete regulatory unit has been defined.

Chromatin studies have produced two further results which are relevant for the potential application of the chicken lysozyme gene locus as a commercially interesting vector system in transgenic chicken. First, the extent of the gene domain is identical in the chromatin of oviduct tubular gland cells and in premacrophages. We conclude therefore that the DNA of the regulatory unit for transcription in myeloid cells also contains all necessary cis elements to direct correct expression in tubular gland cells of the chicken oviduct. Second, no DHS element was found in the transcribed part of the gene locus (Fig. 1A). We conclude that the sequences of the gene proper do not contain any DNA elements for the control of transcription. Replacement of the lysozyme coding region by any appropriate open reading frame would most likely transform the DNA of the gene domain into a vector for high level production of foreign proteins in the egg of transgenic birds.

ACKNOWLEDGEMENTS

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REFERENCES