Transgenic laboratory animals in gene regulation research.

by *Helene Krogh-Pedersen*

Bomholtgård Breeding and Research Centre Ltd., DK-Ry
and Institute of Molecular Biology, Aarhus University, DK-8000 Aarhus, Denmark.

Recently, the use of transgenic animals has found a wide variety of applications in, e.g., developmental issues, the understanding of gene function and the production of proteins of commercial value. Furthermore, transgenic animals are used as models of human diseases. Appropriate gene expression is crucial for most of these applications, and so the importance of the field in which transgenic animals originally were applied, is evident : Gene regulation research, that is the identification of potential genomic regions involved in regulation of genes in higher mammals.

What is gene regulation ?

Generally speaking, all cells in the body contain identical genetic material. However, different cells produce different proteins. *Gene regulation* is defined as the directing of a gene so that it will be expressed in a specific cell type, at a specific level and at a developmentally correct time. Consequently, differentiated cells in eukaryotes possess a remarkable capacity for selective expression of specific genes.

As for protein coding genes, a key aspect in the control of gene regulation is the rate of *transcription*, that is, when DNA is being transcribed into RNA by RNA polymerase II (after which RNA is translated into protein and excreted from the cell). However, gene activity may also be modulated by post-transcriptional and post-translational processes. The most decisive step of regulation is whether or not the gene is at all transcribed. In the present article, transcriptional controlling elements responsible for cell-type specific gene expression will be introduced. This is done from a historical point of view.

Sequences flanking the individual genes control expression

In 1983 several reports presented a major breakthrough in studies of gene regulation (e.g., Walker et al. (1983), Banerji et al. (1983)). At the time, it was possible to detect expression of protein coding genes of higher eukaryotes using a variety of heterologous in vivo and in vitro systems. However, levels of expression were extremely low compared to those observed in differentiated cells in vivo. Remote control DNA-regions stimulating transcription of genes were originally discovered in viral genomes. Now, it was demonstrated, that these remote control DNA-regions were not a viral peculiarity. Thus, the presence of a remote DNA-sequence was found to be essential for gene expression, and furthermore, this DNA-sequence was able to stimulate transcription in a cell-type specific manner. Walker and co-workers (1983) linked putative regulatory control regions from the insulin and chymotrypsin genes to an enzymatic reporter gene (the coding sequence of chloramphenicol acetyltransferase, CAT). Cell-specific expression was then measured by comparison of the expression of CAT in appropriate differentiated cell lines and in inappropriate cell lines. Thus, upon introduction of the recombinant gene into pancreatic endocrine and exocrine cell lines as well as into non-pancreatic cells, not only tissue-specific but cell-specific expression was obtained : In the presence of DNA regions flanking the insulin gene, CAT-expression was seen in endocrine pancreatic cells, whereas DNA regions flanking the chymotrypsin gene directed expression to exocrine pancreatic cells only. No expres-

sion was seen in any other cell lines. The DNA-regions responsible for directing preferential expression in pancreatic exocrine cells were later mapped by deletion analysis (Boulet et al. (1986)). In addition, requirements for specific sequences within these DNA-regions were demonstrated by a decrease in activity as a result of mutations. Banerji and co-workers described a cell specific control element within the mouse immunoglobulin heavy chain gene, which may contribute to the activation of somatically rearranged immunoglobulin variable region genes. The work demonstrated that these newly discovered eukaryotic control sequences may even be located within the gene which they regulate (Banerji et al. (1983)).
A variety of cell-type-specific control elements that stimulate gene expression have since been detected in other cellular genes of higher organisms (reviewed in Maniatis et al. (1987)).

Enhancers

What is described above is the so-called enhancer effect. Thus, the control elements that stimulate expression of specific genes are termed *enhancers*. A general review of enhancers is given by Müller et al. (1988). When linked to a test gene, transcriptional enhancers are able to strongly activate transcription of the gene from its correct initiation site, e.g., the correct position on the DNA strand to start transcribing into RNA. Enhancers are defined as follows.

- They function over large distances of over 1000 basepairs from the initiation site of the gene.
- The activation is independent of orientation of the enhancer.
- The activation is independent of position of the enhancer relative to the initiation site.

Consequently, the enhancer can be positioned either in front of (that is, upstream or 5' of the gene) or behind (downstream or 3' of the gene) the gene or even within the gene, and still, regardless of its orientation, enhance transcription.

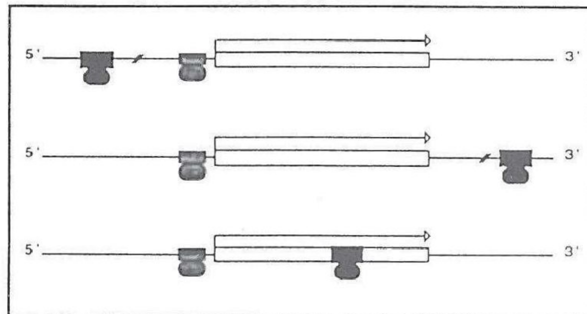
Promoters

Like enhancers, *promoters* constitute gene regulating regions. In contrast to enhancers, promoters are always positioned directly upstream of the coding sequence of the gene. They are typically about 100 base pairs in length. Two types of elements have been identified within the promoter : The *upstream promoter region* and the *TATA box*. The latter is a short DNA sequence motif consisting of T and A nucleotides. It is positioned at a defined orientation, about 30 base pairs from the transcription start site, and is an important promoter component that determines the point of transcription initiation. Some genes do not have a TATA-box. These are the so-called housekeeping genes, which are being transcribed continuously, and at a low level only. As for the upstream promoter elements, e.g., the CCAAT or GC box, they are usually found within a few hundred base pairs from the transcription start site. Generally speaking, the TATA-box determines the exactness of transcription, whereas the upstream promoter elements confer strength and specificity of expression. So, like the enhancers the promoters are able to confer tissue specificity. See Roeder (1991) for a review on transcription initiation.
In summary: The promoter is required for accurate and efficient initiation of transcription, whereas the enhancer strongly increases the rate of transcription from the promoter.

Promoters and enhancers are short cis-acting regulatory elements (that is, they affect the activity only of DNA sequences on their own DNA molecule). *Transcription factors* – also called transacting factors are proteins that by binding to DNA, take part in gene regulation. Both promoters and enhancers are composed of arrays of sequence motifs which function as binding sites for transcription fac-

tors. Thus, Boulets group (Boulet et al. (1986)) and others proposed that differentiated cells contain *transacting factors that interact with cis-acting elements,* thereby mediating gene regulation.

Gene regulation in transgenic animals
When studying gene regulation, a definitive test of cell-specific gene expression requires testing genes in every possible cell-type. Consequently, from the beginning of the 1980s, with the development of transgenic techniques (Gordon et al. (1980), and reviews by Palmiter and Brinster (1986), Jaenisch (1988), Isola and Gordon (1991)), in addition to transfection of DNA into tissue culture cell lines (transient transfection assays), cloned genes have been introduced into the germ line of mice by *microinjection.* The advantages of investigating the regulatory potential of DNA fragments in *transgenic animals* as opposed to cell culture systems, are manifold,



Elements of proximal and remote transcriptional control. While the position of the promoter is always the same, the location of enhancers relative to the transcriptional start site varies. In the top panel the enhancer is positioned in front (upstream or 5') of the gene, in the middle panel behind the gene (down stream or 3') and in the lower panel within the gene. As indicated by the broken lines in the top and middle panel, the enhancer may be found several thousand bases from the gene. An open box illustrates the coding sequence of a gene, and an arrow the direction of transcription (5' to 3'). Black boxes and grey boxes indicate enhancer and promoter respectively. Black circles and grey circles indicate transcription factors that bind to enhancer and promoter regions respectively.

some being the possibility of studying the expression of the transgene in every cell type and throughout development in a genomic context. So too, can a more uniform and biologically correct expression pattern be obtained in transgenic animals. For instance, many human genes have been properly expressed in transgenic mice (Palmiter and Brinster (1986)). This indicates, that the information necessary for specific expression and developmental regulation is present within the transgenic sequences and is able to function in novel locations in the chromosomes of different spices of animals. As a consequence, tests can be carried out of altered position, orientation, or sequence of an enhancer with respect to its ability of directing correct cell-specific expression with appropriate developmental timing. Some studies of gene regulation can be carried out only in transgenic animals and not in cell culture systems. Examples will be mentioned later in this article.
When using transgenic animals in studies of the regulation of specific genes, the approach most often applied is to link regions flanking the transcribed sequences to a reporter gene, and use the constructs for transgenesis (a review on reporter genes is given in Cui et al., (1994)). Tissue samples from the resulting transgenic animals are analysed for expression of the reporter gene. Hence, the expression profile of the regulatory region present in the construct, can be established. Gene regulatory elements can be narrowed down by deletion of fragments until the specific expression disappears, or, if the obtained expression is insufficient and non specific, to include larger genomic fragments until a satisfying expression level is achieved.

Using the approach described above, Ornitz and co-workers (Ornitz et al. (1985)) identified the DNA elements required for pancreas-specific expression of the rat elastase I gene in mice. Later the same group of researchers demonstrated, that the elastase I regulatory region contains a transcriptional enhancer, spanning a region of no more than

(Higgs et al. (1990)), human CD2 gene (Greaves et al. (1989)), chicken lysozyme gene (Bonifer et al. (1990)), mouse metallothionein gene (Palmiter et al. (1993)), human major histocompability complex loci (Chamberlain et al. (1991), human keratin 18 gene (Thorey et al. (1993); Neznanov et al. (1993)), rat whey acidic protein gene (Dale et al. (1992)), rat liver-enriched activator protein gene (Talbot et al., 1994) and recently the chicken β-globin gene (Mason et al. (1995)).

Chromatin takes part in gene regulation

In the eukaryotic nucleus the template for transcription is chromatin, not naked DNA. Nuclear DNA of eukaryotic cells complexed with DNA-binding proteins (other than transcription factors) is known as *chromatin*. Chromatin can be regarded as a highly ordered package of DNA. As the accessibility of the DNA to transcription factors is determined by the gross structure of the chromatin, the ability to regulate chromatin structure is a major component of transcriptional regulation (reviews in Grunstein (1990); Wolffe (1994)). Generally, tissue-specific gene activity is accompanied by an unfolding of the chromatin packaging of the particular gene and an increase in DNase I sensivity. As a result a looping out of the active chromatin occurs, giving transcription factors access to the DNA.

As DNA is not integrated into chromatin in a transient transfection assay, all studies in relation to chromatin structure must involve transgenic animals. Detailed analysis of the mechanisms of regulation in multigene loci has become possible with the development of methods for generating transgenic mice carrying large DNA fragments. The human β-globin cluster has been particularly useful for these types of studies. Functional analysis in vivo based on microinjection techniques and gene targeting by homologous recombination (as in Kim et al. (1992)) make it likely, that there will be significant progress in understanding the regulation of complex gene loci in the next few years.

Just how does LCR function, which mechanism results in position independent and copy number related expression? One property of the β-globin LCR is the ability to induce sensitivity to DNase I digestion over an extensive region of chromatin. LCR is believed to act *first* by organising the entire locus into an open chromatin or DNase I-sensitive domain thereby forming the basis for position independent expression, and *then* serving as a powerful transcriptional enhancer. Notable, the LCR is able to do so even though it is located far from the gene which it regulates. Thus, extensive functional analysis has shown that LCR is a dominant positive activator (or super-enhancer) that acts by forming very stable complexes with genes, to the exclusion of other regulatory elements (Forrester et al. (1989); Ryan et al. (1989); Philipsen et al. (1993)). Functional dissection of the β-globin LCR is in progress. At present, it appears that LCRs do not insulate transgenes from position effects, but rather act as dominant activators by forming stable complexes with genes while excluding other regulatory elements (Kollias and Grosveld (1992); Dillon and Grosveld (1993)).

Additional regulatory elements

Besides promoters, enhancers and LCRs other regulatory elements have been described: *A-elements* (A for attachment) have been found in the chicken lysozyme gene. They map to the 5' and 3' boundaries of the region of DNase I sensivity in the active chromatin, thereby representing the anchorage of the DNA loop domain to the nuclear scaffold (the framework of metaphase chromosomes). A-elements are cis acting elements which form a functional unit and behave like a regulatory insulator. They act as barriers against position effects when placed on each side of a transgene, and are expected to block for activation when positioned between an enhancer and a reporter gene. (Stief et al. (1989); Levy-Wilson and Fortier (1989); Bonifer et al. (1990); Sippel et al. (1992); Chung et. al (1993)). However, inves-

tigations have indicated, that copy-number dependence and position independence may be separated (Thorey et al. (1993)).
Like A-elements, scaffold associated regions (*SARs*) and matrix attachment regions (*MARs*) constitute the anchorage-region of transcriptionally active DNA loops to the nuclear scaffold or matrix (Mirkovitch et al. (1984)). They flank transcriptionally active genes and frequently map within or close to enhancers, LCRs and the DNase I-hypersensitive sites (Gasser et al. (1989). An interesting model involving SARs in chromatin opening was proposed recently (Zhao et al. (1993)). SARs and MARs may act as A-elements and contribute to gene control by forming boundaries between distinct domains in eukaryotic chromosomes, thereby reducing position effects (Phi-Van and Strätling (1988); Stief et al. (1989); McKnight et al. (1992)). However, not all SARs/MARs form domain boundaries. For instance, Thompson and coworkers investigated the role of SAR elements in the regulation of gene expression in a developmental context (Thompson et al. (1994)). When a murine heat-shock-protein gene was flanked by SARs it was expressed in a copy-dependent manner in embryos, but not in differentiated tissues of newborn and adult mice. This suggests a limited capacity of SARs to act as insulators but is consistent with the chromatin opening model proposed by Zhaos group. Consequently, A-elements and SARs/MARs seem to cohabit, although their functions can be separated.
Recently, transcription of the murine immunoglobulin μ heavy chain locus was found to require collaboration between an intronic enhancer and its flanking MARs (Forrester et al., 1994). Unlike the MAR associated with the chicken lysozyme gene, this MAR is implicated in negative regulation by binding transcription factors that participate in repression of enhancer activity in non-B lymphoid cells. Thus, the combination of MAR and the intronic enhancer may constitute a regulatory unit functionally analogous to the LCR in the human β-globin cluster.

This last study illustrates the increasing complexity that researchers of eukaryotic gene regulation are faced with. Ever more complex experimental systems seem to be the most appropriate choice of solution. Some of these are mentioned below.

Future aspects in gene regulation research.
As outlined in this article, considerable advances in describing the molecular mechanism of eukaryotic gene regulation have been made recently. This progress seems likely to continue. Strategies need to be developed to overcome the unpredictable influence of the genomic position effect. By better understanding the mechanisms which cause position effects, it will be possible to develop vector systems allowing random genomic integration of transgenes in such a way that they behave independently of neighbouring DNA. Consequently, efforts are made to solve "The Genomic Domain Transfer Problem", that is to define a genomic domain within which a gene and its regulatory sequences are located (Sippel et al. (1992)). The identification of an A-element, with no influence on transcription itself but capable of insulating any transcriptional unit would be of great importance in the design of transgenes and to molecular genetics as a whole.

Meanwhile other kinds of studies are carried out in the field of future gene regulation research. A design strategy that uses computer modelling to predict how DNA binding domains could be combined to generate novel specificities of transcription factors was reported recently. Hence, *artificial transcription factors* and other DNA binding proteins can be designed by combining known DNA binding domains from different proteins, thereby constructing new fusion proteins (Pomerantz et al. (1995)).
Additionally, the creation of *artificial promoters* based on either artificial or native DNA sequences has now become a reality. It is possible to built a promoter with all the

characteristics desired for a specific study, either by choosing among elements of native promoters or by constructing modules in the laboratory. Grey and collaborators studied the mechanism of transcriptional repressors in the fruit fly (Drosophila) by creating a promoter with synthetic binding sites for transcription factors (Grey et al. (1994)). A model is presented, in which activators and repressors co-occupy neighbouring sites in a target promoter, and the repressors are shown to block the ability of the activator to contact the transcription complex. In another study, two heterologous prokaryotic activators were found to activate transcription synergistically from an artificial promoter bearing binding sites for both proteins, suggesting that bacterial RNA polymerases interact with more than one transcriptional activator at some natural promoters (Joung et al. (1994)).

So far only Drosophila and prokaryotic artificial promoters have been produced, but artificial eukaryotic promoters will no doubt follow and it will certainly initiate a new era in molecular genetics.

Summary

The knowledge of how genes are regulated in mammals has increased enormously since the development of the first transgenic mice in the early 1980s. Up to then, studies of gene regulation were carried out in cultured cell lines. The advantages of investigating gene regulation in transgenic animals as opposed to cell culture systems, are manifold, e.g, allowing the study of expression of the transgene in every cell type and in relation to development.

The term gene regulation implies that genes, present in all cells in the body, are directed to expression in specific cell types, at a specific level and at a developmentally correct time. This is brought about by the influence of DNA regions flanking the individual genes. Thus, the tissue specificity of a gene is defined by the surrounding DNA regions.

Some of the types of DNA regions which are responsible for the expression pattern of a specific gene, are named promoters and enhancers. The promoter is positioned in front of the gene close to the transcription start site, and is responsible for correct transcription initiation. The enhancer strongly enhances the transcription from the promoter, and is able to do so regardless of its postion and orientation. Consequently, the enhancer can be positioned in front of, behind or within the gene which it regulates. Both promoters and enhancers are able to bind transcription factors, which are proteins that by binding to DNA, take part in gene regulation.

In recent years, studies carried out in transgenic mice have made it possible to identify new types of control regions in addition to promoters and enhancers. Thus, the locus control regions (LCR) from the human β-like globin locus is able to confer tissue specific, position independent expression of human β-globin genes in transgenic mice. It is likely that LCRs from different genes will become important in gene rgulation studies in transgenic mice, as position independent expression of transgenes may be obtained by including an LCR in the microinjected construct. However, so far LCRs have been characterized in only a small number of genes.

Individual transgenic mice produced by microinjection carrying identical transgenes, express the transgenes at highly varying levels, depending on the site in the genome at which the transgene has integrated. This is due to the position-effect, that is, the expression of the integrated transgene is influenced by the surrounding DNA. Hopefully, in the future, a better understanding of the mechanisms causing the position-effect, will lead to the development of vector-systems that allow transgene expression which is independent of the site of integrations.

Resumé

Udviklingen af transgene teknikker har haft afgørende betydning for hvad der i dag vides om genregulation i højerestående dyr. De første transgene mus blev lavet i begyndelsen af 1980erne. Indtil da foregik studier af genregulation i cellelinier. Fordelene ved brug af transgene mus frem for cellelinier er mange. Eksempelvis kan nævnes at: 1) alle tænkelige celletyper kan analyseres for ekspression af RNA eller protein (DNA-koden oversættes til en RNA-kode (transkription) som igen oversættes til protein, der kan udskilles fra cellen), 2) ekspression i relation til udviklingen kan undersøges, 3) der opnås mere ensartede og biologisk korrekte ekspressionsniveauer.

Gener, som er til stede i alle kroppens celler, styres til udtryk i specifikke celletyper på bestemte tidspunkter og på et bestemt niveau. Dette sker ved, at flankerende DNA sekvenser indvirker på hvert enkelt gen. Således styres ekspressionen af hvert enkelt gen, hvorved genets vævsspecificitet kan defineres.

De styrende DNA-regioner opdeles bl.a. i promotere og enhancere, hvor førstnævnte typisk er beliggende umiddelbart foran det gen hvis udtryk den styrer. Promoterens opgave er at sørge for, at transskriptionen starter på det rette sted på DNA-strengen. Modsat kan genets enhancer, som virker ved kraftigt at forøge transscriptionsraten af genet, befinde sig langt væk fra genet og i alle orienteringer – dvs. foran, bagved eller endda midt i ge-

net. Promoter- og enhancer regionerne binder proteiner kaldet transscriptionsfaktorer, som er specifikke for de enkelte celler. Herved indgår transscriptionsfaktorerne i genregulationen. Indenfor de seneste år, har studier i transgene mus gjort det muligt at identificere typer af kontrolregioner, som er forskellige fra promotere og enhancere. Således har locus kontrol regionen (LCR) fra det humane β-globin locus vist sig at resultere i vævsspecifik og positions-uafhængig ekspression af humane globin-gener i transgene mus. Fremover kan locus kontrol regioner fra forskellige gener måske få stor betydning i forbindelse med genregulerings-studier, idet positions-uafhængig ekspression af transgener kan opnås ved at inkludere genets locus kontrol region i det mikroinjecerede DNA-konstrukt. Endnu er locus kontrol regioner dog kun karakteriseret i nogle få gener. Når transgene mus produceres ved hjælp af mikroinjektion, opnås ekspressionsniveauer som varierer meget fra den ene transgene mus til den anden, idet niveauerne er afhængige af hvor i musens genom, integrationen af transgenet finder sted. Dette fænomen benævnes positions-effekt, og skyldes, at transgenet påvirkes af omkringliggende DNA-regioner. Målet for mange studier indenfor genregulation har netop været, at opnå en bedre forståelse af de mekanismer som fører til positions-effekten. Håbet er, i fremtiden at kunne udvikle vektor-systemer, som tillader tilfældig integration i genomet, således så transgenet udtrykkes uafhængigt af omgivende DNA-sekvenser.

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University of Copenhagen & National University Hospital

Department of Experimental Medicine

Panum Institute
Blegdamsvej 3
DK- 2200 Copenhagen N
Denmark

Phone: +45 35 32 73 73
Fax: +45 35 32 73 99

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