led us to include these chapters in this review. The three final chapters outline studies on transgenic farm animals in particular with regard to utilization for production of pharmaceutical proteins (Wilmut et al.), modification of important production traits (Ward and Nancarrow) and attempts to improve disease resistance (Müller and Brem).

In this review we have not addressed the topic of oncogenes and transgenic mice, which undoubtedly is of increasing importance for cancer research, nor the use of transgenic mice as disease models. Also, studies on mammalian development with the help of transgenic animals were not included. Results of such studies may well affect applications and strategies in animal breeding some time in the future.

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Transgenic regulation in laboratory animals

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Abstract. This chapter is an attempt to summarize some commonly accepted and some more subjective opinions about the regulation of transgene expression in laboratory animals. After a short historical introduction, I present some general notions regarding gene structure/function. The spotlight shifts then to the description of the most popular techniques for gene transfer, including the targeted gene replacement. The different approaches are briefly discussed in terms of intrinsic advantages and limitations regarding gene expression patterns. Furthermore, the role of enhancers, promoters and other *cis*-acting elements such as silencers and dominant control regions as well as their involvement in the chromatin on-off state are discussed on the basis of a specific example studied in our laboratory. The review concludes by presenting recent results and the new perspectives opening in the field of 'surrogate' (also called 'reversed') genetics. Some problems which remain to be solved both at the technical as well as at the social-ethical level are also briefly presented.

Key words. Transgenic mice; microinjection; recombinant DNA; gene expression; transcription factors; chromatin; homologous recombination; episomal maintenance; embryonic stem cells; germ line; position-effect; mosaicism; globin genes.

Why and how did it all start?

It is a common belief that direct genetic transformation of increasingly complex organisms by the genetic carrier material has received more serious attention only since the classical experiment of Avery and colleagues[2]. I would like to go beyond this rather scholastic belief by daring to say that this has been the pervasive dream that has accompanied all studies in the field of modern genetics. Direct genetic transformation relies on the possibility of physically breaking the barrier of the compartment in which the genetic material is normally stored (i.e. the cell or the nucleus) with chemical, physical or biological tricks and depositing new, and possibly defined, genetic material into the host genome. In this first phase one is mainly confronted with the technical issue of efficiency of the process of gene transfer. Down the road, other important problems such as the stability and the faithful expression of the newly introduced genetic information arise and, as we shall see, several related aspects have yet to be completely elucidated. It is only after solving the majority of these problems that we can exploit the technique of direct genetic transformation in its full potential, that is to obtain useful information about specific gene expression patterns and their complicated network of primary and secondary metabolic effects in the context of the entire organism. Beside the mere academic interest, the application of direct gene transfer for the 'improvement' of the genetic repertoire of economically important animal and plant species also seems to receive increasing attention (see chapters by other contributors to this multi-author review).

In the last few decades, the booming of techniques allowing rapid and easy cloning of genes from various sources, along with a formidable progress in basic embryo culture procedures and manipulation (refs 12 and 50, and references therein) have provided the tools for the modern transgene techniques. In fact, several very early attempts to transform entire organisms had failed, probably due to the rather primitive stage in the techni-

cal handling of macromolecules[33, 34, 40, 67, 104, 124]. For the very same reason, reports describing the successful transformation of vertebrate organisms with cloned DNA molecules appeared almost around the same time[13, 24, 44, 92]. In these very early attempts the first barrier (i.e., the efficient introduction and integration of defined DNA in the host genome) had just been timidly crossed, and already the next important questions had emerged: (a) were the newly introduced sequences faithfully transcribed in the host organism? and (b), if so: can a consistent expression pattern be obtained with virtually every gene fragment? Before trying to answer these questions we will open a short parenthesis to analyze the current knowledge about eucaryotic gene structure and function.

Genes, promoters, enhancers, chromatin and gene expression

As shown in figure 1, a typical eucaryotic gene consists of various operationally distinguishable portions. We shall list them according to the approximate chronology of their discovery and/or experimental definition: (a) a coding portion (*structural gene*) which is only partly preserved in the mature transcripts (the exons, which give rise to leader, open reading frame and trailer of the mature mRNA, and the introns which are spliced out during the RNA maturation process); (b) proximal *cis*-control elements (the *promoter*, a region surrounding the transcription start site, which specifies quantity, accuracy of initiation and polarity of transcription); (c) more distal *cis*-elements (the so-called *enhancer* or *silencer* elements, which, in co-operation with the promoter, specify the rate of transcription), and (d) additional remote control elements (dominant control regions (DCR) and perhaps others) which may specify the overall on-off state of the chromatin containing and surrounding the transcribed unit. Further explanation of the terms and a corresponding list of essential literature are mentioned in the legend of figure 1.

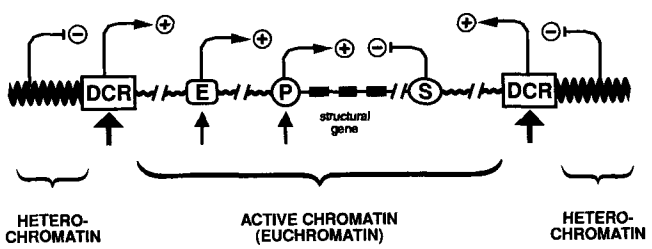

Figure 1. Elements controlling specific gene transcription in chromosomes. The scheme is a general representation of the distribution, location and operating mode of various *cis*-elements flanking a putative regulated structural gene. The general signs '+' or '−' following *bent arrows* indicate a respectively positive or negative effect on transcription rate of the locus. Symbols: *Thick wavy line*, condensed chromatin (operationally defined as nuclease-resistant; the chromatin features and analysis are reviewed by refs 16, 21, 100, 114, 128); *Thinner wavy line*, chromating displaying moderate nuclease sensitivity; *Straight line and bars*, structural gene; *Interruptions* in bars and wavy lines, undetermined size of the segment; *Vertical arrows*, regions of more pronounced DNase hypersensitivity; DCR, dominant control regions[10, 28, 43, 47, 49, 110, 123]; E, enhancer[77, 101]; P, promoter[29, 74]; S, silencer (Baniahmad et al.[4], and references therein). The position and relative importance of each element can vary among different genes (except for the promoter, which by definition is close to the transcription start). In this model, the DCR elements are displayed as barriers between euchromatin and heterochromatin. It must be noted that this is only a working model, based on current, commonly accepted knowledge, and that no definitive proof exists for the precise role of each element. In particular, it has not yet been established, whether the DCR elements really function as insulators for the propagation of heterochromatin.

The position and the importance of each general element varies enormously from gene to gene (see below). Studies involving the reintroduction of intact and altered gene fragments in cultured cells or in animals suggested a hierarchical path of dominance going from DCR (d) to structural gene (a). For instance, one could clearly show that in cell cultures some promoters work efficiently only when strong enhancers are located in *cis*[29, 77, 101], and that some enhancer-promoter combination may require additional information before being efficiently expressed when re-inserted at an inappropriate position in a host chromosome (see below). The notion that each of these broadly defined elements (promoter, enhancer, or other remote *cis*-control regions) can display distinct tissue- and/or developmentally-specific preferences introduces us to the next chapters in which we shall see some possible consequences of the alteration of the natural order and combination of these components during the generation of transgenic organisms. So far, specific gene transcription has been a major concern, and this is justified by the difficulties in recapitulating tissue-specific transcription patterns of many gene fragments. Nevertheless, one has to bear in mind that a number of additional important regulatory steps exist (splicing, transport and stability of mRNA, translation efficiency, stability, trafficking and posttranslational modification of polypeptides) which have been found to be partly tissue-specifically modulated. These topics become more and more important in experiments in which inter-species barriers are crossed or in which gene products should be generated outside their normal developmental compartment[71] or in competition with resident components[20, 136].

Available techniques, fate of newly introduced DNA

The most straightforward method to introduce defined DNA in the nucleus is microinjection. In order to generate a transgenic organism, it is necessary to perform the injection into the pronuclei of fertilized eggs (Hogan et al.[53], and references therein). The manipulated eggs are then transferred to the oviduct of foster mothers where they are allowed to develop to term. Although we still lack a lot of information regarding the mechanism of integration of the foreign DNA into the host chromosome, the usual assumption is that this event relies on the stochastics of the normally occurring chromosomal breakage and repair[53, 93]. In order to generate homogeneous integration patterns, this event must occur very

early. When the random integration occurs at later stages, we obtain a chimeric animal in which the transgenic cells are confined to some developmental compartment or cell lineage. This occurs rather frequently in the generation of transgenic mice whereby up to 25% of the founder animals may be mosaic to some extent [53, 93] (our unpublished results). In the generation of transgenic frogs, chimerism is the governing rule, for it is not possible to inject the DNA in the pronucleus, and one has to rely on the uptake of the cytoplasmically injected DNA during early cleavage [92, 93].

When generating transgenic mice, the percentage of manipulated eggs which develop to term and the percentage of transgenic animals among the newborns depends largely on the skill of the experimenter and the quality of the equipment. Under optimal conditions about 30% of the manipulated eggs should develop to term and 10–15% of them may be transgenic [53, 93]. To conclude, about 2–3% of the microinjected eggs will yield the desired product, that is: an animal which carries the injected sequences, usually organized in a tandem array of variable size, integrated at an unpredictable location in one chromosome. If the animal is not mosaic, all its cells will contain the newly integrated gene cluster in the same position and in the same chromosome. The randomness of the integration site between different transgenic lines is a problem which significantly disturbs the analysis of the phenotypes generated with this technique (discussed below). Another major consequence is that when integration occurs at a 'permissive' location in the host chromatin (means 'not in heterochromatic regions') the insertion frequently disturbs a resident locus [48, 55, 96, 107, 127, 133, 135]. This is the reason why transgenic lines can only rarely be propagated in homozygous state, thus requiring the tedious monitoring of the pedigree of heterozygous transgenic strains.

Alternative ways of transferring DNA into whole organisms include the injection of (or infection by-) viral particles in developing embryos [53, 55] or the use of totipotent embryonic stem cells [53, 55, 58]. The first technique often results in strong methylation of the inserted sequences and the consequent loss of expression of the linked gene [55]. This hypermethylation is apparently occurring at lower rates when the DNA is injected in pronuclei (Palmiter and Brinster [82], and our unpublished results). The use of e.g. engineered retroviruses leads to severe limitations of the size of the transgene segment [55, 82]. Nevertheless, this technique has at least one advantage in that it usually results in single copy integration events since it exploits viral integration mechanisms.

A certain number of transgenic lines has already been generated with the help of pluripotent embryonic stem cells (ES cells, Evans and Kaufman [32]). In this approach, the first step is the permanent transformation of cultured ES cells with conventional gene transfer techniques (refs 8, 46, 90, 97, 103, and references therein). The transformed cells are re-introduced in early blastocysts, where they contribute to the development of a chimeric embryo after re-implantation in foster mothers. The major difference between this technique and the pronuclear microinjection lies in the fact that there is a pre-selection of the desired genotypic or phenotypic configuration (i.e., at least the dominant selection marker which accompanies the gene of interest must be active, thus implying that the gene cluster is not integrated in constitutive heterochromatin). By far, the most appealing feature of the ES mediated gene transfer, is the possibility of targeting the transgene construct to a pre-determined chromosomal position via homologous recombination (refs 17, 56, 130, 138, and references therein). This technique allows the very precise analysis of specific alterations of the genetic material, without suffering the limitations of the more conventional transgene technique. Nonetheless, the culturing and maintenance of fully totipotent ES cells is still technically rather demanding, and this is the reason of the rather meager publication list concerning the successful germline propagation of homologous gene transfers [60, 98, 118, 119, 137]. This is the case in spite of the fact that the separate key techniques (homologous recombination and successful generation of mice transmitting specific ES genotypes in the germline) have been fully developed since a couple of years.

In fact, several groups still prefer the conventional pronuclear microinjection of specific DNA fragments, and the scientific community feels the need of even simpler techniques for gene transfer. In this respect, a sudden burst of interest has been raised by the claim that efficient gene transfer could be obtained by pre-incubating sperm with DNA and using the manipulated gametes for in vitro fertilization [65]. Unfortunately this result remains so far unsubstantiated, but one cannot exclude that some other groups may have already identified the crucial factor(s) which permitted the originally claimed high frequency in gene transfer. In fact, it appears as if this technique might be more readily applicable to the genetic transformation of larger mammals (M. Birnstiel, personal communication).

Even simpler approaches have been proposed recently. For instance, it has been demonstrated that it is possible to inject DNA directly into muscles and obtain expression of the foreign reporter gene [132]. Finally, specific DNA can potentially be targeted to various somatic tissues with the help of recently developed membrane-receptor-mediated gene transfer techniques. These techniques have been pioneered with the model system exploiting internalization processes of the transferrin receptor [126]. The concept of specific receptor-mediated gene transfer can apparently be extended to other types of membrane receptors such as T-cell specific receptors (M. Birnstiel, personal communication). These two last techniques are not aimed to the stable propagation of the altered genotype through the germ line, but must be regarded as having a potential application to some kind of somatic therapy, and have been mentioned here since

they will require extensive studies with laboratory animals in the future.

Factors and sequences influencing the predictability of transgene expression patterns

Changes of trends in studying expression of transgenes

A stable transgenic mouse line (i.e., in which the foreign gene is inherited in a Mendelian fashion) is of limited use if the foreign gene is not expressed. In this case the foreign DNA element is expected to generate at most some insertional mutants, which result in autosomal recessive lethal or sub-vital mutants. Several groups have exploited this observation and analyzed further the sequences flanking the insertion, thereby identifying the candidate genes for several pathologies[48, 55, 96, 107, 127, 133, 135]. After the pioneering phase (1980–82) the improvement of technical knowledge (see chapters below) led very soon to an explosion of scientific publications emphasizing the correct expression of a particular transgene combination (see fig. 2).

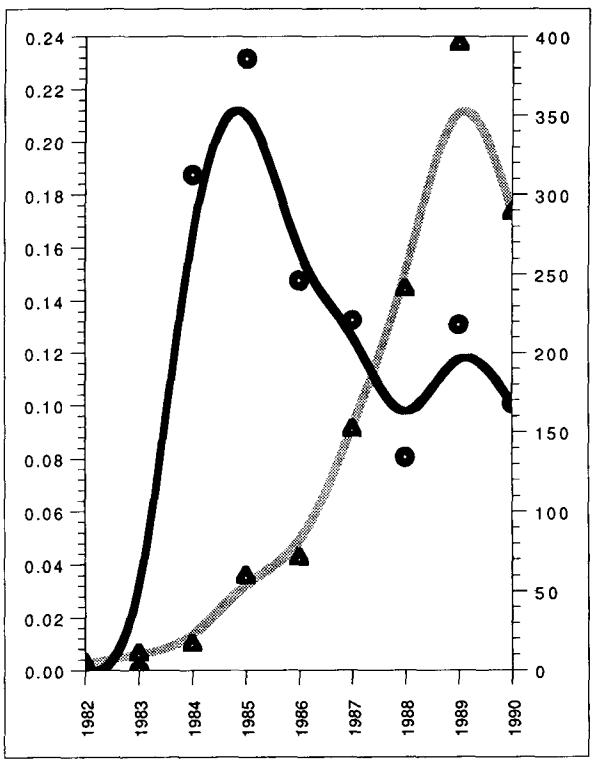

Figure 2. Statistics of publications dealing with transgenics and with tissue-specific expression patterns. Citations were searched in the Database MEDLINE from 1980 to date. Search queries were: 'tissue-specificity, gene expression, transgenic'. Triangles and circles indicate real values and lines represent a mathematical smoothing of the profiles (obtained with the program WingZ for MacIntosh). The values for 1990 were extrapolated from data between January and May. *X-axis*: year of publication; *Right Y-axis* (referring to triangles and shadowed line), number of documents dealing with transgenics; *Left Y-axis* (referring to circles and black line), proportion of documents dealing with tissue-specific transcription pattern in transgenics. As pointed out in the text, the amount of documents follows an exponential profile which may already have reached a plateau (trend is not clear, remembering that values for 1990 are not definitive), whereas the proportion of papers dealing with tissue-specific expression had a pronounced peak (close to 25%) between 1984 and 1986 and seems to be stabilizing around 10% in more recent times.

In this type of experiments, the typical constructs consist of a specific promoter or enhancer region, flanked by variable amounts of specific DNA and linked to a reporter gene such as CAT[70, 134], β-galactosidase[66, 122] or the homologous gene from a different species or a modified homologous gene[63, 82, 93]. The proportion of scientific publications emphasizing the tissue-specific pattern of expression of the transgene reached some 25% in 1985 and after a sharp decrease seems to stabilize around 10% of the publications dealing with transgenic organisms. The reason for this reduction in the direct interest on transcription regulation of the transgene (i.e. the use of the animal as an extension of the expression assays in cell cultures) has probably to be sought in the more exciting perspectives which are opening now in the field. Perhaps some authors may have had to struggle more and more to publish papers dealing with this kind of issue which, in the eyes of some editors, is getting 'out of fashion', as is the fate with several topics in experimental science. Furthermore, one realized very soon that, with some notable exceptions (discussed in the paragraph dealing with DCR elements), the information relating to transcription regulation does not exceed that obtained with refined cell-culture systems and well-designed expression assays. To conclude, it appears that the trend is now going more towards the study of the physiological effects and consequences of newly introduced genes or mutations, rather than just measuring the transcription rate of different promoter or enhancer mutants.

Procaryotic sequences and inter-species barriers

Amplification in bacteria is the only available convenient way to obtain the desired recombinant DNA on a preparative scale. This requires the linkage of the sequences of interest to a prokaryotic replication vector (usually a plasmid). During the initial gene transfer experiments one invariably used linearized or circular, plasmid-linked genes. Some early experiments had already shown that procaryotic sequences might have a strong negative effect on the function of some genes[86, 92]. It is not too speculative to affirm that, in the enthusiasm of that time, these early warnings were royally ignored by virtue of some other reports in which plasmid sequences did not seem to play a major role[15, 83, 89, 116]. Ironically, our group followed the mainstream, although previous experiences from our own laboratory[86, 92] were extremely explicit in disclosing pBR sequences as undesirable in eucaryotic expression systems. It was only after several years of frustration that it was commonly accepted that the procaryotic moiety might have played an undesired inhibitory role regarding the expression of some transgenes. In fact, it was only upon removal of most of the procaryotic moiety that several gene fragments, like the human and murine β-globin[117], the alpha-fetoprotein genes[63, 78],

and muscle-specific genes[102], to name a few, displayed the expected expression pattern. It must be noted that the inhibitory function of procaryotic sequences cannot be generalized, since some relatively large procaryotic segments like the β-galactosidase coding sequence seem to leave some eucaryotic promoters or enhancers undisturbed[122], while interfering with other ones, like the metallothionein promoter[82, 113].

In this context, it must be pointed out that even eucaryotic sequences can behave in an unexpected manner when combined to other eucaryotic fragments. To name the most dramatic examples, segments from the SV40 virus genome or from the mouse metallothionein gene promoter have been reported to exert epistatic effects on certain (but not all) genes[82, 115, 121]. The nature of these un-anticipated effects is still unknown, but one can speculate that these phenomena are due to some cryptic or fortuitous silencer elements present in the disturbing sequences or generated at the junction of the normally un-linked segments. In fact, the erratic influence of plasmid sequences of the expression of transgenes could similarly be explained by the presence of fortuitous co-dominant silencer sequences in the procaryotic DNA. According to this model, the negative effect could be overwhelmed by the concomitant presence of some (but not necessarily all) strong, *cis*-positive-regulatory segments (see fig. 1) or by long stretches of naturally flanking sequences (see next paragraph). Undesired effects caused by cryptic sequences were anticipated when using genes from different species. In the initial stage of experimentation, genes from heterologous species were often used to generate transgenic animals, since they allow easy identification in the host organism. These experiments outlined very well that the much-feared inter-species barriers are probably not a major disturbance for the efficiency and accuracy of expression of transgenes (refs 58, 82, 96, and references therein). This observation suggests that in the course of evolution several basic gene control mechanisms must have been tightly conserved both at the *trans*- and the *cis*-level.

Role of specific enhancers, promoters and dominant control regions: the example of the murine alpha-globin genes

Enhancers and promoters consist of clustered binding sites for specific transcription factors. The number, identity and geometric arrangement of the various sites determine the strength and tissue selectivity of each cluster. For instance, one has identified enhancers or promoters which work with different efficiencies and display distinct preferences when tested in lymphoid cells[45], liver cells[78], or erythroid cells[36, 41]. The primary evidence is usually obtained by transfection of reporter genes driven by the specific enhancer or promoter (or combination) in different cultured cell lines[29, 74, 77, 101]. The specific expression pattern usually matches the predicted pattern (i.e. the one characteristic to the donor gene segment), and sometimes also correlates with the presence/absence of rate-limiting non-ubiquitous transcription factors[29, 74, 77]. It has been a great achievement to demonstrate that at least some of these *cis*-elements were both necessary and sufficient to fully support a complex developmental program accompanying final differentiation. This is even more surprising since the control elements were sometimes disconnected from their cognate structural gene and, on top of that, inserted at a random location in the chromosomes of the host-organism. In fact, remarkably 'small' segments of the immunoglobulin genes[15, 89, 94], lens crystallin gene[81], alpha-fetoprotein genes[63], homeobox gene[122] or pancreatic genes[116] are capable of faithfully emulating the developmental expression program of their endogenous counterparts. Does this mean that complex developmental patterns do not require large genomic portions and, consequently, do these observations revive the debate around the C-value paradox[69]? This is contrasted by the notion that several other genes require much larger portions of flanking regions to be faithfully expressed in the host organism.

This fact is best illustrated with our early attempts to obtain faithful expression of murine alpha globin genes. Encouraged by the initial reports dealing with immunoglobulin genes (see references above) we undertook a while ago a project aimed at the transcription analysis of newly inserted alpha globin genes in the mouse. The mouse alpha 1-globin gene was modified by swapping the 3'-untranslated trailer with that of the rabbit beta gene, thus rendering it distinguishable from the endogenous gene at both the RNA and DNA level (fig. 3, top and middle)[93]. A 10 kb genomic segment harboring the marked alpha globin gene (fig. 3) was used for the initial series of pronuclear microinjections.

This 10 kb segment was microinjected either as purified fragment, or linked to plasmid sequences, foreign transcriptional enhancers, etc. (details about constructs are given in table 1). More than 30 independent transgenic lines were generated and none of them was found to express the transgene in detectable amounts in erythropoietic tissues (see examples in fig. 3 and summary in table 1). In parallel, a cosmid clone was isolated via hybridization with the alpha 1-coding sequence. This genomic segment spanned more than 40 kb and included beside the alpha 1-globin gene, a second alpha globin gene (at a distance of 20 kb downstream) and a third cross-hybridizing segment (upstream), tentatively assigned to an embryonic gene (fig. 3, top)[93]. The alpha 1-globin region of the cosmid was substituted with the marked gene and the modified construct was microinjected in pronuclei. Five transgenic founder mice were identified, of which two were mosaic and did not express the transgene (see RNA analysis of mice Nr. 275 and 277 in fig. 3), while the remaining three lines efficiently expressed the marked alpha 1 globin gene (see RNA analysis of mice Nr. 256, 274 and 278 in fig. 3 and summary in table 1).

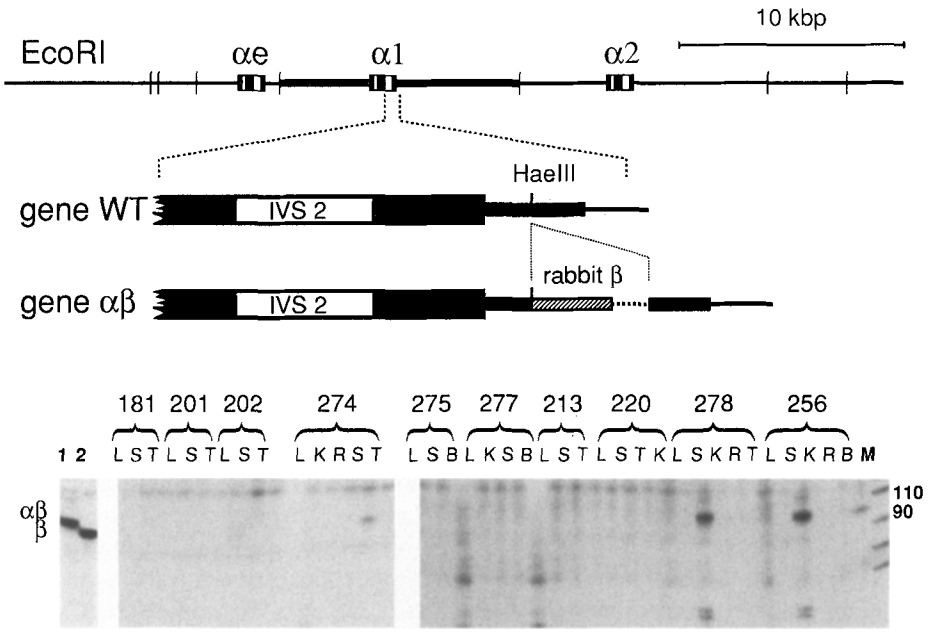

Figure 3. Tissue-specific expression of marked murine alpha-globin gene in transgenic mice. *Top*: map of the cloned murine alpha-globin locus harboring three alpha globin genes[93]. *Vertical bars* indicate the position of EcoRI restriction sites. Structural genes are symbolized by *filled and empty rectangles* (respectively, exons and introns). *Bar at the top right* indicates the scale corresponding to 10,000 base pairs (10 kbp). The 10.5 kbp EcoRI fragment used to generate many transgenic lines and harboring the marked alpha 1 gene is indicated with the *thicker line*. Symbols: WT, wild type, IVS2, second intervening sequence of mouse alpha-globin. *Middle*: Scheme of modification of the murine alpha 1 globin gene. A rabbit beta-globin 3′ sequence was inserted in the 3′ sequence of the alpha-globin gene as indicated. The recombinant gene was called 'alpha-beta' and this alteration allowed the easy analysis of the transgenic sequence at both the DNA and the RNA level[93]. The alpha-beta gene was used to generate transgenic lines either in the 10 kbp context or when reimplanted in the 40 kbp context (see also table 1 for more results). *Bottom*: Analysis of RNA. RNA from various tissues was isolated and subjected to RNase protection analysis as described[93]. The antisense RNA probe used matched the trailer of the rabbit beta-globin gene (see above). Three-digit numbers refer to mouse lines, letters indicate the organ from which the RNA was extracted. A characteristic RNase-protected fragment of 100 nucleotides is expected upon specific hybridization (see control lanes at left) of the transgenic product with the probe. Lanes 1 and 2 are control analyses in which either native rabbit globin mRNA was probed (lane 2, 10 ng rabbit globin mRNA) or the total RNA obtained from cells transiently expressing the alpha-beta construct (lane 1). Symbols: L, liver; S, spleen; K, kidney; T, testes; B, bone marrow; R, brain; M, size marker (position of 90 and 110 nt fragments is given at right), $\alpha\beta$, position of protected fragment upon hybridization with RNA from recombinant gene; β, position of protected fragment obtained with native rabbit globin RNA. Bone marrow RNA from mice Nr. 275 and 256 was slightly degraded. In general 10 µg of total RNA were used for analysis, except for bone marrow from which only 2 µg were used. Analysis displayed here includes mice which are transgenic for different construct types (see table 1 for more details): Mouse Nr. 181 is a transgenic from series Nr. 3 (10 kbp fragment + pBR), mice 201 and 202 from series 4 (10 kbp fragment + MT), mice 213 and 220 from series Nr. 5 (10 kbp fragment + SV40 + TK + pBR) and the remaining from series Nr. 6 (40 kbp fragment + SV40 + TK + pBR). In the latter series, faithful and abundant transcription is obtained in at least three transgenic animals in spite of the presence of plasmid sequences in the transgene. The specific expression pattern in mice 256 and 278 could be demonstrated later to be heritably stable (data not shown).

Table 1. Expression of marked transgenic alpha-globin gene is dependent on the size of the cotransferred flanking sequences.

Nr.	Transgene structure		Number of mice		Expression	
	Segment size	Linked elements	Born	Transgenic	Unspecific	Specific
1.	10 kb	None	54	4	0	0
2.	10 kb	SV40 + TK	48	9	0	0
3.	10 kb	pBR	56	5	0	0
4.	10 kb	MT	35	4	0	0
5.	10 kb	SV40 + TK + pBR	62	11	1	0
6.	>40 kb	SV + TK + pBR	23	5	0	3
Total		–	278	38	1	3

The table summarizes the results from 38 transgenic lines in which a marked murine alpha 1-globin gene (fig. 3) was inserted as a DNA segment of various size and linked to different DNA elements. Line 6 displays results from experiments in which a modified cosmid insert was used for microinjection. Unspecific expression in the mouse line of the group 5 was in the brain. Specific expression was measured in spleen and/or in bone marrow (fig. 3). As outlined in the text, consistent expression of the transgene in hematopoietic tissues was only detected when the inserted fragment contained substantial amounts of native flanking sequences. This was observed in spite of the presence of procaryotic sequences in the transgene. Details of the alpha globin locus and the modification procedure are given in figure 3. Symbols: TK, herpes thymidine kinase truncated gene; SV40, simian virus 40 enhancer[3]; MT, mouse metallothionein gene promoter; pBR, procaryotic plasmid sequences; kb, 1000 base pairs.

With this observation we could demonstrate that something essential to the autonomous 'unfolding' of the developmental potential of the alpha 1-globin transgene must be contained in the additional 30 kb of the cosmid insert. Meanwhile, similar results have also been reported for the beta globin genes[49,123], immunoglobulin light chain genes[43], some plant genes[28], the lysozyme gene[110], and T-cell specific genes[47]. In some of these cases, the subsequent analysis has led to the narrowing of this 'helper' function to some relatively small DNA segments located in the remote flanking regions. These segments correspond to regions of the chromatin which are markedly accessible to nuclease digestion (see fig. 1, and references in the legend). Since these segments confer a predictable expression pattern to the linked reporter gene, these segments are often referred to as Dominant Control Regions (DCR)[10,28,43,47,49,110,123]. Still very little is known about the exact mechanisms governing this phenomenon. A number of experiments suggested that these regions might be involved in attachment to the nuclear matrix, thereby allowing the reorganization of the linked gene into active chromatin and insulating it from the general negative effects of the new flanking sequences[10,110,123]. In some instances it could be shown that upon linkage to DCR, a transferred gene is expressed at a level which is proportional to the number of integrated copies[110], thus implying that the tandemly arranged genes are functioning *independent* from each other when separated by the DCR segments. This is usually not the case, neither in transgenic lines[27] nor in permanently transformed cell cultures (Stief et al.[110], and our unpublished results). It is also not clear, whether the position relative to the coding body of the gene has any significance, since in the experimental situations the DCR can be placed at very variable positions in the construct (C. Bonifer, personal communication). However, it is still not clear whether these regions really possess some general 'insulating' properties and most importantly, whether they can be fully distinguished from conventional, strong and tissue-specific transcriptional enhancers. From a more pragmatic point of view, the availability of such DCR elements represents an important tool to reduce the variability of expression patterns which characterizes transgenic lines obtained by conventional microinjection techniques[1,10,111]. For the time being, this seems to be the only available technique to obtain reproducible expression patterns when the transgene is re-introduced via the conventional microinjection technique.

Extrachromosomal maintenance, heterologous versus homologous integration

We should point out that the maintenance of the transgene in an episomal vector could also isolate it from undesired and unpredictable *cis*-dependent effects. Unfortunately, and in spite of many dramatic efforts, attempts to assemble stably propagating episomal vectors for transgenic mice have so far only led to unreproduced claims[59,87]. Particular molecules, like the polyoma virus or the bovine papilloma virus could be demonstrated to propagate autonomously in transgenic animals, but unfortunately the normal cell metabolism is disturbed due to the requirement of transforming functions for plasmid maintenance[31,62,64]. It is noteworthy to mention at this point that a very recent claim[108], if substantiated, may revive the hopes of the specialists, since a Japanese group has reportedly succeeded in maintaining stable episomal non-transforming DNA molecules over several generations of transgenics[108].

Furthermore, the erratic expression patterns in the transgenic organism can be minimized if the recombinant construct is inserted at the homologous locus. Although also in this case some epistatic effects cannot be completely excluded, the integration of cloned DNA into its homologous location offers the unique possibility to obtain consistent phenotypic and genotypic changes[17]. This approach relies on the concomitant success at three key levels: (a) integration of the foreign DNA via homologous recombination of flanking sequences in pluripotent embryo stem cells; (b) the successful reimplantation of the manipulated ES cells into a host blastocyst and the formation of a chimeric host; and (c) the contribution of the manipulated ES cells to the germ line tissue of the developing host. Several groups have been successful in the first two steps but only a handful of reports exists which describes the successful germ line transmission of the altered genotype. This lack of efficiency seems to be related to the delicate metabolic and physiologic balance which underlies the maintenance of pluripotency of the ES cells during the culture period necessary for the genotypic manipulation (Capecchi[17], and references therein). We can only hope that these technical difficulties will be solved in the near future, thus rendering the technique of targeted gene replacement more broadly accessible. Specific gene replacement, along with the refinement of other more conventional transgene approaches, will pave the way to new exciting perspectives in the study of developmental regulation of gene expression.

The new challenge: the surrogate genetics of the nineties

The concept of 'surrogate genetics' had first been introduced in 1977 by Birnstiel and Chipchase[9] to describe the approach of modifying cloned genes in vitro and testing their function after re-introduction into a living cell (at that time, the frog oocyte). Now this concept can be extended to entire and complex multicellular organisms and the term 'reverse genetics' has also been proposed for these studies[130]. A breakthrough in this sense is presented by the possibility of specifically interfering with master regulatory pathways which govern developmental programs. A large array of genes encoding primary and secondary regulatory proteins have been isolated. This palette includes for instance genes of ubiquitous and

Table 2. Physiological effects of transgenes.

Nr.	Alteration	Type	Consequences	Examples
1.	Random integration of cloned genes	Gain-of-function, dominant, <100% penetrance	Growth	A
			Tumors/neoplasia	B
			Differentiation	C
			Secretion	D
			Rescue of null alleles	E
2.	Constitutive agonists/or antagonists	Loss-of-function, gain-of-function dominant, <100% penetrance	Antisense RNA	F
			Ribozymic RNA	G
			Neutralizing antibodies (FAB)	H
			Interfering subunits	K
			Overexpression/Bypasser	L
3.	Site-directed toxins	Loss-of-function, dominant <100% penetrance	Cell ablation	M
4.	Integration at homologous locus	Loss-of-function, or change-of-function, recessive, 100% penetrance	Gene disruption	N
			Targeted alteration	O
5.	Integration at random locus	Loss-of-function, recessive, 100% penetrance	Gene disruption	P
			Gene alteration	Q

The table resumes in five categories the most common ways to introduce a transgene which results in a physiologically or metabolically measurable effect. other explanations are in the text. Key to the letter code for references describing the listed examples or related reviews: **A**, (examples: 83, 45); **B**, (reviews: 51, 23, 111), (examples: 14, 8, 64, 131); **C**, (reviews: 110, 130), (examples: 89, 15, 94, 43, 7, 134); **D**, (review: 20), (example: 136); **E**, (examples: 6, 25, 88, 72, 105, 7, 118); **F**, (reviews: 52, 129), (examples: 57, 112); **G**, (working in cell cultures and in vitro, reviews by: 19, 85). **H**, (working in cell cultures: example 18); **K**, (example: 109); **L**, (examples: 61, 76, 54, 73, 95, 117); **M**, (examples: 30, 11, 75); **N**, (review: 17), (examples: 137, 60, 119, 98); **O**, (example: 118); **P**, (reviews: 107, 48, 55), (examples: 96, 133, 127, 135); **Q**, (possible, but no examples found).

cryptic transcription factors[29,74], nuclear and membrane-bound receptors[5,37,38,68,79,91,106], oncogenes and anti-oncogenes[22,23,51,124], growth factors and lymphokines[80,84], homeotic genes[39,58,99,130] etc. For many of these regulatory gene products a rather detailed picture of their basic molecular functions and structures already exists. Still, the missing link is the definition of their role(s) in the network of decisions governing the processes leading to terminal differentiation of the various tissues. To this aim, three major experimental approaches have seen the light in the past few years: (a) the loss-of-function assay, (b) the change-of-function assay, and (c) the gain-of-function assay. The concept underlying these strategies is essentially the same: to try to generate a genetic configuration such that a single, specific master regulatory function is altered, and verifying the consequences of this alteration in the developing organism. There are several alternative ways to produce such alterations which have been tested and found to be promising to varying degrees (see table 2).

The most attractive way of obliterating a specific function is certainly to eliminate the coding gene via homologous recombination with a construct carrying a null mutation (insertions, deletions, stop codons or others, see examples at point 4 in table 2). This is achieved best in pluripotent ES cells, as described before. The alteration is usually expected to be a recessive mutation and must therefore be bred to homozygosity. The mutation is expected manifest itself with 100% penetrance. In any case, this system is only applicable when the gene of interest is unique und when the mutation is not metabolically lethal. Other ways to inactivate or reduce the activity of determined gene products involve the use of specific overexpressed anti-sense RNA molecules (antisense RNA, table 2-F) or, more elegantly of RNA molecules which can be used to specifically cleave mRNAs (ribozymes, table 2-G). The disadvantage of these methods is the requirement of a very abundant expression of the RNA antagonist, before a reduction of the specific mRNA is achieved. For this reason, results in transgenic organisms have been so far only very limited in number (see table 2, legend).

Some more sophisticated approaches exploit the concept of trans-dominant mutations in which a protein antagonist is generated. This can be either a constitutive inhibitor which binds to the same target as the wild type, but without eliciting the same response[35] (table 2-K), or a null-allele which can inhibit the action of the wild type via hetero-multimerization[26,42,109]. In some instances it has been shown that deregulated production of intact regulatory gene products is sufficient to produce interesting phenotypical alterations (table 2-L). In this case, the most spectacular example is certainly the recent success in overriding the sex determination program in female mice by forced expression of a transgene encoding the *Sry* product[61]. For obvious reasons, this technique will only be applicable to subset of regulatory proteins which have the appropriate prerequisites, such as a multimeric structure.

Finally, it is possible to bring the genes encoding regulatory functions under the control of foreign promoter/enhancer/DCR combinations and obtain their ectopic expression in the transgenic organism (i.e. in tissues where the gene product is normally absent or present in reduced amounts). This approach is bound to give sometimes erratic results due to the intrinsic variability of expression

patterns discussed in preceding paragraphs, but can help in giving a molecular explanation of the restricted expression pattern of some key regulatory proteins. This approach has already allowed a better understanding of several phenomena related to growth control, establishment and maintenance of tumors, achievement of differentiated states, secretion pathways (see examples A–D in table 2) and has been also used to demonstrate the rescuability (table 2-E) of specific genetic defects.

Concluding remarks

Palmiter and Brinster concluded their review in 1986[82] by presenting a set of crucial problems related to gene transfer techniques in organisms. We may look at this list again, in the light of what has been discussed in this chapter, and witness the progress of this last half-decade:

1) *Improvements in the efficiency and ease of introduction genes into the germ line of animals.* Technical progress has only been marginal, and the success of the procedure still largely depends on the skill of the experimenter.

2) *Development of simple means of introducing single copies of genes, but without the limitations of current retroviral vectors.* No convincing alternative has been proposed so far, except perhaps for gene transfer via electroporation in ES cells, which results in fewer integrated copies per genome[53].

3) *Discovery of methods for insulating transgenes from the effects of neighboring chromatin.* Progress in this area is very promising, especially since the discovery of DCR elements (see previous paragraphs).

4) *Development of techniques for homologous recombination, so that endogeneous genes can be deleted or replaced.* As discussed before, targeted gene replacement is still a technically demanding technique, but since the ice has been broken, we expect reports of substantial improvements in the efficiency of this approach in the near future.

5) *Improvement of methods for phenotypic inactivation of gene expression (e.g. development of effective antisense RNA constructs).* This field is under intense investigation and since a large number of regulatory factors seem to work as part of multimeric units, it is possible to add also the techniques of trans-dominant protein agonists and antagonists.

6) *Development of inducible promoters that will permit experiments in which gene expression can be tightly controlled.* This is still the black sheep of modern molecular biology. In spite of the fact that several inducible transcription systems have been described, none of them offers the sufficient guarantees of a very low basal level and a satisfactory expression in the induced state. Furthermore, it is difficult to find inducible systems which would not overlap with normal physiological functions. One possibility will be to employ ligand-dependent modules derived from a completely unrelated species. For instance, we are trying in our laboratory to combine the ecdysone binding domain (M. Lezzi, M. Imhof and S. Rusconi, unpublished results) to drive other nuclear receptor functions such as the glucocorticoid or the estrogen receptor[5].

This means that the progress in the last half decade has been slow but still very promising and has solidified some of the crucial weak points. Many experiments which had been nearly unthinkable five years ago are now within our reach. The possibility of re-shaping an organism by changing its genotype in a site-directed fashion, means the ultimate opportunity to understand the laws governing the storage, retrieval and interpretation of the marvelous code which is the source and the consequence of the life process itself. A better understanding of this intricate web of catalytic and stoichiometric interactions will pave the way for a more integrated view or new concepts heading toward the diagnosis and maybe even a somatic therapy of heritable diseases. Other perspectives include the possibility of making a more rational use of our natural resources, be them bacteria, plants or animals, and to better exploit natural processes, rather than using complicated and polluting machines for the production of sophisticated substances.

Nobody can tell us whether our society is prepared to use this knowledge in an appropriate manner, and historical experience may induce someone to be rather pessimistic. This is the reason why these techniques have attracted an intense, sometimes malicious, controversy around some truly ethical and other pretended-to-be-so arguments as well as around some more or less realistic scenarios. As said just above, there is still a lot to experiment with, and, provided our efforts can be better explained to and understood by the public opinion, we will try to solve these problems, aided by our ever-young good students and their enthusiasm, and by a generous gulp of much-needed optimism.

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