Multiple Strategies for Gene Transfer, Expression, Knockdown, and Chromatin Influence in Mammalian Cell Lines and Transgenic Animals

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Abstract

Manipulation of the eukaryotic genome has contributed to the progress in our knowledge of multicellular organisms but has also ameliorated our experimental strategies. Biological questions can now be addressed with more efficiency and reproducibility. There are new and varied strategies for gene transfer and sequence manipulation with improved methodologies that facilitate the acquisition of results. Cellular systems and transgenic animals have demonstrated their invaluable benefits. In this review, I present an overview of the methods of gene transfer with particular attention to cultured cell lines and large-scale sequence vectors, like artificial chromosomes, with the possibility of their manipulation based on homologous recombination strategies. Alternative strategies of gene transfer, including retroviral vectors, are also described and the applications of such methods are discussed. Finally, several comments are made about the influence of chromatin structure on gene expression. Recent experimental data have shown that for convenient stable transgene expression, the influence of chromatin structure should be seriously taken into account. Novel chromatin regulatory and structural elements are proposed as an alternative for proper and sustained gene expression. These chromatin elements are facing a new era in transgenesis and we are probably beginning a new generation of gene and cancer therapy vectors.

Index Entries: Gene transfer; gene expression; antisense; transient transfection; stable expression; retroviral infection; gene therapy; microcell fusion; position effects; chromatin; Locus Control Region; insulator; artificial chromosomes; recombination systems; viral vector.

1. Introduction

Starting with recombinant DNA technology, a large spectrum of applications has emerged. In particular, intense efforts were made to transfer genetic information to cell lines, primary cell cultures, diverse organisms and tissues, or in the generation of genetically modified organisms. Gene transfer technology requirements vary depending on the transfer method and cell type. Without a doubt, gene transfer has been critical in the advance of our knowledge related to general phenomena such as gene regulation, posttranslational events, recombinant protein production, and gene therapy.

One particular fact to be taken into account is that there is no general methodology for gene transfer. Each cell type and organism needs prior careful characterization to ensure optimal transfer conditions to reach the highest efficiencies and reproducibility in terms of gene expression. But this is not the only obstacle that we face when we decide to transfer genetic information. In the majority of experimental biological systems, we are constantly confronted by two variables. The first one

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has to do with the great variability in the expression levels of transgenes, and the second with a less studied phenomenon, the progressive extinction of transgene expression, which is present in the majority of the cases ***(1,2)***. In addition, a less clear general phenomenon has to do with multiple copies of the same transgene that, once integrated into the host genome, induces a phenomenon called co-suppression in plants, which causes gene expression silencing when multicopies of transgenes are integrated in tandem ***(3)***.

In the present article, I summarize the experimental strategies and applications of recombinant DNA transfer. I analyze the most general methods for gene transfer, taking the two major experimental strategies to study transgene expression into account; that is, transient strategies (also called episomal), including virus vectors and strategies of integration into the host genome. Chromosomal transfer and artificial chromosomes will also be included in this overview as a strategy to study gene expression in a natural chromosomal environment. I discuss transgene expression in detail and its association with regulatory elements, and conclude with some prospects and a recently discovered way to possibly overcome some transgene expression difficulties.

1.1. Goal of Recombinant DNA Transfer

The lack of accessible in vivo systems obliges the search for alternatives to gene expression in eukaryotic cells. During the past years a large list of gene transfer applications has arisen and the constant appearance of new methodologies has made gene transfer more accessible and reproducible for research scientists.

1.1.1. Regulation of Gene Expression

One of the most common applications for gene transfer is the study of gene expression patterns. Developmental studies can be carried out in different types of cultured cell lines or in transgenic animals (discussed later). Those studies can be supported by the use of primary cell cultures from various organisms and tissues, but we can also take advantage of transformed cell lines derived from viral infections or even different types of tumors. Overexpression of gene products can be an alternative to define gene function, to interfere with and search for a particular signal transduction pathway, or even to titrate posttranslational modifications of an endogenous peptide. At the present time, gene transfer methodologies allow controlled levels of gene expression, although some problems remain unsolved.

Gene regulation studies are probably the most frequent way to investigate the activity of eukaryotic gene regulatory elements. Actually, the list of such elements is still growing, with classic examples being promoters, enhancers, locus control regions (LCRs), and, more recently, insulators ***(2,4,5)***. All of these studies are based on two main components—the use of measurable reporter genes and subsequent cell transfer in a transient or stable way. Plasmids carrying different reporter genes, like chloramphenicol acetyltransferase (CAT), and more recently luciferase genes, β-galactosidase and the green fluorescence proteins, are actually commercially available, and they facilitate and are less time consuming when we are interested in defining the activity of a control element (**Table 1**). Such reporters are frequently used in transient transfection experiments when the activity can be assayed enzymatically or colorimetrically between 24 and 48 h post-transfection. These kinds of experiments are not restricted to the use of those reporters. Specific genes can be used with particular developmental and differential expression patterns. One of the classic examples emerged from the use of the human adult β-globin gene in the presence of its own promoter and with distinct versions of the LCR ***(6,7)***. In this case, no enzymatic activity can be followed but molecular biology protocols can be applied like reverse transcriptase–polymerase chain reaction (RT-PCR), RNase protection, S1-mapping, or even Northern blotting to estimate transcription levels ***(8)***.

It is very important to mention at this point that transient transfection experiments, although they are very instructive, can give inconsistent results. In particular, this happens when compared to the same reporter vectors integrated into the genome

Table 1
Common Reporter Genes

Gene	Detection
β-galactosidase (β-gal)	Enzymatic and colorimetric
Chloramphenicol acetyl transferase (CAT)	Enzymatic and radioactive
Luciferase	Luminiscence
Green fluorescent protein (GFP)	Fluorescence

of the host cell. Therefore, the chromatin environment of an integrated reporter plasmid can give totally different results compared to episomal vectors. Also, chromatin structure needs to be remodeled to allow regulatory factors to recognize their target sequences. Nonetheless, there is direct and indirect experimental evidence suggesting that circular test plasmids acquire a chromatin-like organization with nucleosomes positioned along the plasmidic DNA every 200 bp or more, instead of the usual 147 bp ***(9)***. Indirect evidence has shown that the use of histone deacetylase inhibitors in transiently transfected cells can cause the reactivation of silenced reporter genes ***(10)***. In any case, the use of transiently transfected vectors represents a useful and direct way to analyze the activity of regulatory elements on reporter genes. Yet in the integrated context, where chromatin plays a central role, permanent and more realistic results are obtained.

1.1.2. Peptide Production

Actually the generation of peptides from an isolated cDNA has many possibilities for diverse applications. One of the principal applications of peptide production is the use of tagged sequences to assist in the purification of a particular fusion protein for subsequent immunizations to generate antibodies for co-immunoprecipitation experiments. The use of eukaryotic cells can be particularly attractive when we take posttranslational peptide modifications into account. Technically, transfection conditions for protein production should be performed in medium supplemented with serum or in medium without serum. However, it is preferable to express proteins in a serum-free medium, as serum proteins interfere with the subsequent purification protocols of the expressed protein. A long list of research topics can take advantage of such experimental strategies, ranging from cell membrane receptors to signal transduction and chromatin structure. Recombinant peptide expression and maturation can also be useful for transport studies coupled with immunolocalization. More recently, a number of improved methods for particular peptide production have emerged. Among them, insect (bacculovirus), *Drosophila* cell lines, and bacteria are alternative options to mammalian cell lines, with a direct impact on the cost and on the amounts of recombinant peptides that are produced.

1.1.3. Antisense Expression

A more recent and effective way to manipulate gene expression is by the transfer of expression vectors that transcribe and target specific antisense RNAs to interfere with gene translation ***(11,12)***. This approach allows the study of gene function in a more natural context, but several examples have shown a significant cellular cytotoxic effect ***(13)***. A recent publication argues in favor of the use of short interfering RNAs (siRNAs) between 21 and 25 nucleotides ***(14,15)***, but one of the encountered difficulties is the transient effect of such interfering RNA sequences. More recently, Brummelkamp and colleagues ***(12)*** designed a novel mammalian interference expression vector that requires a specific 19-nucleotide sequence in the context of a well-defined transcription start site and a highly specific stem–loop structure under the control of the H1-RNA gene promoter. The advantage of this pSUPER–siRNA vector is that it can be integrated into mammalian cells, thereby ensuring a constant rate of interference.

These gene transfer strategies based on RNA interference represent, at least at the level of cultured cell lines, an alternative to knockdown gene products as an experimental strategy to address reverse genetic questions ***(15,16)***.

1.1.4. Tissue-Specific Expression

One of the principal goals in gene therapy design is the definition of the activity of highly specific regulatory elements in terms of their particular patterns of gene expression. Transient and stable transfections have demonstrated their utility, but with some limitations. At the organism level, transgenic animals and targeting strategies are also very instructive. The definition of tissue-specific patterns of gene expression remains crucial from the viewpoint of not only understanding the development of an organism, but also for future gene and cancer therapy protocols. At this point, it is critical to understand gene expression profiles but it is urgent to mention that it is not a simple task as a result of the different networks and redundancies used in nature to reach a highly specific pattern of gene expression. If we add the complexity and the participation of cell nucleus compartments and their associated chromatin structures, it remains clear that a lot of work and new tools are needed to start manipulating the genome ***(17,18)***.

2. Methods for Gene Transfer

2.1. Transient Transfection

Successful transient transgene transfer is generally subject to several parameters. The gene transfer method, the cell physiology and cell type (suspension or adherent cultures), the enzymatic or colorimetric method of reporter activity quantification, the DNA amount, and the data normalization all require particular attention. The time period of gene expression in transient transfections ranges from 24 to 72 h. The cell conditions are critical for success in terms of healthy and actively dividing cells being best as efficient recipients of recombinant plasmids. Optimal conditions can be reached using a high-quality plasmid purification procedure. In our hands the best method is the one offered by Qiagen. Transient transfection efficiencies and reproducibility can be determined by taking advantage of a second reporter that expresses an easily detectable and measurable gene product. Such a reporter gene should not be present in the genome of the cell type to be tested, or in the worst case, present and expressed at low levels. The most commonly used reporter genes are shown in **Table 1**.

It is important to outline that plasmid integrity and its ability to reach the nucleus drastically diminishes the efficiency of reporter gene expression ***(19)***. Southern blot analysis of 24 and 48 h posttransfection plasmids demonstrated that almost 50% of transiently transfected circular vectors are degraded (Recillas-Targa, unpublished observations). Long discussions have been recurrently addressed through the years concerning the location of transiently transfected plasmids inside the cell or the cell nucleus. Transient transfection and reporter gene expression inside the cell remains a controversial issue. Gene transfer methods usually direct plasmid DNA to the cytoplasm and it is not clear what percentage of these circular or linear molecules reach the cell nucleus. In addition, careful parameter definition is needed to reach a reasonable degree of reproducibility. It has been suggested that, among different mechanisms, passive diffusion of plasmids through the cytoplasm and nuclear pores represents the way in which reporter genes can migrate to the cell nucleus and be transcriptionally activated. New evidence argues in favor of a novel insight to explain the transfer of a DNA plasmid to the cell nucleus based on import DNA signals ***(19,20)***. This new proposition is established through the idea of protein–plasmid DNA interactions in the cytoplasm, and that such transcription factor interactions mediate nuclear localization signals, allowing the importation of exogenous DNA into the nucleus.

If this vision is true, we now face an additional obstacle because the transfected plasmid will be conditioned to the tissue- and stage-specific factors and their associated nuclear import signals. One clear conclusion is that the procedure to analyze the expression of a transiently transfected

gene can be oversimplified and we can face misinterpretations.

2.1.1. Lipofection

Cationic liposome-mediated transfection (lipofection) has been a more recent and efficient method for gene transfer both in vivo and in vitro ***(21,22)***. Positively charged cationic liposomes form complexes with the negatively charged DNA molecule, allowing their fusion to cellular membranes. This complex is able to enhance gene delivery in diverse cell types, tissues, and even in combination with viral particles ***(23,24)***. Furthermore, in vivo, cationic liposomes have been used successfully to deliver genes into different tissues including lung, endothelium, and muscle, as well as for drug and vaccine delivery ***(25,26)***. Lipofection is routinely performed in the absence of serum directly in the cell culture media. At the present time a growing list of commercial and noncommercial liposome formulations are available ***(27,28)***, outlining those that can be used in the presence of serum, which facilitates the overall procedure (like Effectine from Qiagen). Particular attention is needed to establish the liposome-to-DNA ratio for each cell type and DNA molecule. An excess of cationic liposomes could be toxic for a particular cell line, an observation that suggests us to fix a low amount of liposomes and gradually increase the plasmid DNA concentration. Additional care is needed during the coupling procedure, as it needs to be done very slowly with addition of progressive amounts of DNA to wrap each liposome particle with the largest amount of plasmid DNA. In this way, optimal transient or stable transfections can the reached. Finally, in our hands lipofection has yielded the best results when adherent cells are used.

2.1.2. Particle Bombardment

Biolistic technology has been born as an alternative to gene transfer, particularly based on the need for plant transformation. In this procedure, target cells and tissues are held under vacuum for bombardment at variable distances. The DNA to be transferred is usually coupled to gold particles and bombardment occurs in a chamber that absorbs the shock waves and gases of the gunpowder explosion. The most recent modification to biolistic technology is the use of a helium-powdered acceleration system. Gene transfer has been widely used in plants, for example, maize suspension cells and embryos ***(29,30)***. This method of gene transfer is not restricted to plants and there are examples of *in situ* transfections in animal tissues ***(31)***. It is worthwhile to mention that Bio-Rad offers the best technology and variety of instruments for this procedure.

2.1.3. Electroporation

An alternative and widely used method for gene transfer is based on the delivery of an electrical pulse that decays exponentially through time. These electrical pulses permeabilize cell membranes, allowing DNA molecules to be transferred inside the cell. Electroporation, as a strategy for gene transfer, involves several parameters that require particular attention, forcing the researchers to calibrate each experiment as a function of the DNA molecules to be transferred, and in particular, for the cell type to be transfected. The electrical pulse for an optimal transfection needs to take into account the cell diameter or size and the composition of the cell membrane. In addition, it is well known that the electrical pulse induces a high degree of cell death because of the discharge. Of course electroporation conditions should be established carefully and for each cell type to find equilibrium between the highest efficiencies of gene transfer and the minimal amount of cell death. More recently, a new design of electroporation apparatus allows in vivo transfection in whole-embryo culture. This improvement represents an attractive alternative for *in situ* gene expression, particularly during embryonic stages, which certainly complements genetic approaches like the generation of transgenic and mutant mice ***(32)***.

2.1.4. Direct DNA Injection

Direct naked DNA transfer to cells has been widely used. It remains clear that the delivery to specific cell types remains limited with this strategy. Nonetheless, the direct delivery of plasmid

DNA has been successfully applied to gene transfer into muscle cells, and particularly into epithelial cells ***(33,34)***. One clear limitation of this procedure is the lack of control of critical parameters like plasmid DNA concentration, degradation, and efficiencies of gene transfer. Nonetheless and for specific applications like ectopic gene transfer, direct naked DNA transfer demonstrates some advantages.

2.2. Stable Expression

I have discussed previously the advantages of the generation of stable cell lines. Briefly, for the generation of stable lines, the transgene of interest should be co-transfected with the same circular plasmids or separate plasmids carrying a selected marker gene that gives drug resistance to select positive integrants. At present a growing list of selected marker genes are available. Nonetheless for every stable transfection assay, transgene integration into the target cell should be carefully monitored. For example, when the transgene of interest and the selected marker are transfected on separate plasmids we can obtain resistant cells but without integration of the test plasmid. Another scenario is the integrity of the transgene. We have observed that in the range of 1 to 10%, integrated vectors are not intact, showing deletions that hamper their further analysis. In addition to all these careful characterizations of the integrant genes, Southern blot analysis can be very helpful to determine the copy number of the integrated transgenes. This could be valuable information depending on the type of experiment planned. In addition, antibiotics or drugs required for selection need to perform in a dose–response curve to determine the optimal concentration for each cell type and for each particular cell culture condition. One of the most frequently used marker genes is the amninoglycoside phosphotransferase gene (APH or neo^r), which confers resistance to the geneticin selective antibiotic (G418 sulfate) by directing the synthesis of the APH enzyme that renders the drug inactive through phosphorylation.

In a typical stable transfection experiment, we first need to allow the cells to recover from transfection for 24 to 72 h in the absence of selection. This permits the cells to express adequate amounts of the resistance enzyme to protect the stably transfected cells against the drug. Then, 48 to 72 h after transfection the culture media should be removed and replaced with fresh media complemented with the drug. Selection can pursue for 1 to 3 wk posttransfection with frequent changes of the selected culture media. Finally, the integrity and copy number should be determined by Southern blotting.

An easy alternative is the generation of stable pools that consists of groups of stable lines derived from different integration events of a particular transgene. With this strategy, we do not analyze one integration event, but instead we measure a group of events that reflect the overall activity of the transgene. This is a less time-consuming strategy compared to the selection of individual clones, which for some cell lines is difficult, particularly when they are not able to grow in semisolid media where clonal selection is carried out.

2.3. Pronucleus Microinjection and Knockout Technology (ES cells)

Transgenic animals have been created with the aim of studying gene function and generating models of gene expression patterns during development, and to correlate dose observation with diseases. Transgenic animals, in particular mice, are unique for their production of peptides in an in vivo context, particularly in milk. Transgenic animals still have several technical limitations, starting with the low frequencies in obtaining founder animals. More recently, site-specific homologous recombination systems have allowed the elimination of a given sequence from the mouse genome or the site- and time-specific gene targeting of foreign sequences, and this has become a more frequently used experimental strategy ***(35,36)***. On the other hand, inducible systems like tetracycline are more commonly used to control the activation of gene expression in transgenic animals at a particular moment, eliminating the risk of lethal phenotypes.

A more commonly used strategy for the manipulation of the mouse genome is by the introduction of mutations into embryonic stem (ES) cells by homologous recombination and the subsequent generation of chimeric mice. In this way, the mouse genome can be manipulated in favor of the loss of developmental gene expression patterns (for a review, *see* **ref. *37***). Two main limitations that have emerged are first, the early lethal phenotypes, making the targeting approach very difficult to evaluate. Second, detailed analysis in a particular cell lineage can be very difficult, as some phenotypes affect multiple tissues in the mouse. At present, particular attention has been focused on the generation of conditional manipulations of the mouse genome to avoid the previously mentioned problems, and this allows phenotype characterizations in the cell or tissue type of interest at a specific developmental stage ***(38,39)***.

2.4. Artificial Chromosomes and Transgenesis

In the course of the past two decades a remarkable effort had been made in the development of new strategies for gene expression that incorporate large genomic sequences. Scientific development has clearly established that in mammals the complexity of their genomes needs to incorporate large genomic sequences to transgenic cells or animals to be able to recapitulate endogenous gene expression patterns. Thus, at present it is clear that knowing the coding sequences of genes remains insufficient to define their time- and tissue-specific expression. This is mainly because there are more and more evidence showing that genes require a sophisticated set of proximal (promoters) and long-distance regulatory elements to achieve their proper gene expression ***(6,18)***. Therefore, the use of artificial chromosomes from yeast and bacteria has been an attractive alternative, not only to recapitulate endogenous gene expression patterns, but even more interestingly to manipulate large genomic regions taking advantage of homologous recombination strategies ***(40–42)***. Thus, transgenesis with artificial chromosomes has proven to be useful in the study of a variety of regulatory and developmental processes, but also for biomedical and biotechnological applications ***(41)***.

Two classes of artificial chromosomes type vectors are commonly used with large cloning capacities as a main feature: the yeast artificial chromosomes (YAC) and the bacterial-derived bacterial artificial chromosomes (BACs) or P1 artificial chromosome (PACs). The YAC and BAC/PAC vectors permit the incorporation of genomic inserts ranging from 100 kb to more than 1 Mb. The insertion of such large genomic sequences favor the inclusion of all the regulatory sequences needed for the faithful gene expression. Furthermore, the incorporation of all the elements required for gene expression certainly contributes to ensure position-independent, copy number-dependent and optimal levels of transgene expression. But one of the most attractive and useful features of artificial chromosomes is the apparent unlimited capacity to generate a large variety of modifications that can be introduced to such vectors. That includes target disruption of specific sequences, inversion, or even insertions, all of them maintaining intact a large portion of the genomic sequence of interest. Therefore, specific genomic sequences contained in a YAC or BAC/PAC can be manipulated but at the same time the wild-type genomic milieu is to some degree conserved. Such scenario will probably allow collection of more realistic results, facilitating an accurate interpretation of the obtained data.

2.4.1. Yeast Artificial Chromosomes

Yeast artificial chromosomes are eukaryotic cloning vectors that are able to stably integrate DNA fragments up to 1 Mb ***(43)***. They contain all functional elements for their incorporation in yeast cells as artificial chromosomes and they have been suitable for the generation of stable cell lines and transgenic animals ***(42)***. Concerning the YACs, some technical considerations need to be outlined. For example, regarding the manipulation and microinjection of YAC DNA, the pres-

ence of ionic strength (100 m*M* sodium chloride) has been demonstrated to be taken into consideration with the aim to stabilize YAC DNA molecules in solution. Furthermore, the addition of polyamines prevents shearing of such large DNA molecules ***(44)***. The YACs are generally of the same size range as the endogenous yeast chromosome, thus for their isolation it is recommended to transfer the YAC into a distinct yeast strain where the artificial chromosome can be easily separated by electrophoretic techniques. With regard to these technical considerations the efficiency on the generation of transgenic animals with YACs is similar to that obtained with standard DNA constructs, although the amount of YAC DNA molecules microinjected is much less because of the large size of the YAC vectors. In general, transgenic animals generated with YACs carry single or less than five copies of the transgenes integrated to their genome. But the main aspect to be taken in consideration is the evaluation of the YAC transgene integrity ***(45)***. Systematic survey of the YAC transgene is needed, as deletions, inversions, or mutations can be incorporated during yeast manipulation or when the YAC DNA is isolated and purified before microinjection. Southern blot, PCR amplification, and even *in situ* hybridization are recommended to evaluate the integrity of the transgene.

2.4.2. Bacterial Artificial Chromosomes

Bacterial artificial chromosomes are circular plasmid DNA molecules that are hosted in *Escherichia coli* and they can incorporate approx 300 kb of DNA. In comparison to YACs, BACs and PACs are much more stable and have been also used for the generation of transgenic cells and animals, including mice and zebrafish (***40***, and references therein). The BAC/PAC transgenic animals have also been prepared by the generation of ES stable cell lines by co-transfection along with a selectable marker, followed by the production of chimeric mice ***(46)***. Unfortunately and as in the case of YACs, rearrangements, deletions, and insertions can also occur in BACs and PACs. Therefore, a detailed and careful analysis of the integrity of the inserted sequences is also needed. At this point, we can say that BAC/PACs are in some aspect quite similar to the YACs, although for the analysis of relatively small sequences (<100 kb), BAC/PACs seems to be the choice. In contrast, for lager sequences, up to 1 Mb, YACs are more convenient.

In terms of the advantages or disadvantages of one or another type of artificial chromosome, the use of YACs offers a more flexible and reliable way to control sequence modifications based on the efficient and elevated rate of yeast homologous recombination. The YAC sequence manipulation can also be performed by replacing the targeted sequences with specific yeast selectable markers surrounded by known homologous sequences. Such strategy has been widely used to introduce mutations at precise locations followed by the excision of selected markers. For the BACs, the sequence engineering using homologous recombination has not been as direct and several groups of investigators have invested in the generation of innovative methods to manipulate BAC/PAC sequences ***(47,48)***. At present, YACs are much easier to be modified in comparison to BAC/PACs vectors, but at the same time they are more difficult to handle. On the other hand, and as mentioned, BAC/PACs are much more stable than YACs. Thus, there are no convincing reasons to favor one type of artificial chromosome from another.

2.4.3. Examples of the Use of Artificial Chromosomes

To illustrate the utility of artificial chromosomes I decided to present some examples where large genomic sequences are used. One of the initial studies was the generation of a 130 kb YAC transgene incorporating the imprinting *Igf2/H19* locus ***(49)***. Artificial chromosomes had been extensively studied for β-globin studies, in particular for chromatin domain organization and regulation of gene expression. For example, studies of the human β-globin locus gene regulation during development introduced specific sequence modifications (mutations) by homologous recombination techniques in yeast cells followed by the generation of YAC transgenic mice ***(50)***. It

became clear that this type of strategy brings several attractive advantages, in particular because it incorporates the great majority of the genomic sequences needed for proper developmental regulation of the multifamily β-globin group of genes. Thus, the obtained results where close to what could be predicted that is occurring in its natural context. Furthermore, some of the modified transgenes using artificial chromosomes turned out to be very informative because they can recapitulate similar or equivalent genetic abnormalities found in patients affected by diverse forms of β-thalassemia diseases *(51)*. In terms of the β-globin locus regulation, a large number of distinct genetic modifications have been generated and analyzed using YAC transgenes containing the human β-globin locus *(52)*. For example, deletion of components or the entire LCR had been generated and their effects analyzed with respect to the globin regulation of gene expression during development *(50)*. Additional manipulations included the removal of regulatory sequences, interreplacement and alteration of gene order, and orientation of key regulatory elements like the LCR *(53,54)*.

In summary, artificial chromosome transgenesis had demonstrated its impact in the studies related to the regulation of gene expression, complex systems like the mammalian central nervous system, imprinted loci and multifamily gene domains like Hox and globin genes. The size of genomic inserts, ranging from less than 100 kb to more than 1 Mb, in optimal conditions allows the incorporation of most, if not all of the regulatory components relevant for the appropriate spatial and temporal gene expression. It is clear that the benefits of artificial chromosomes transgenesis will be incorporated in biotechnology processes such as the production of peptides with pharmaceutical interests.

3. Chromosome Transfer

3.1. Microcell Fusion

Because of the central role of chromatin structure in the regulation of gene expression, undoubtedly genetic studies need to be done in the context of the intact chromatin environments *(6,17,18)*. Homologous recombination and transgenesis have been demonstrated as powerful tools for modifying and manipulating mammalian genetic loci. The majority of such studies are performed by microinjection of transgenes into the pronucleus of a mouse-fertilized oocyte, or by the use of mouse embryonic stem cells where specific mutations or deletions can be generated by homologous recombination. In both cases we are confronted by two main problems. First, both approaches are limited by their low frequencies and large screening procedures are needed, and second, we are restricted to some mammalian organisms, particularly mice, limiting the manipulation of the human genome in a more natural chromatin environment. To overcome such problems, intact chromosomes can be transferred from one cell type to another, allowing their genetic manipulation *(55–57)*. Complementary to such chromosomal transfer, we can manipulate the transferred chromosome in the chicken B-cell line, DT40, which is an avian leukosis virus (ALV) transformed cell line where high targeting efficiencies by homologous recombination can be reached (ranging from 10 to 90%) *(59)*. Much less is known about the molecular mechanisms for the unusual high efficiencies of homologous recombination seen in this chicken cell line. The critical point with the use of the DT40 cell line is that chromosomes from other species can be transferred into these cells, where targeting efficiencies of the transferred chromosomes are of an unusually higher magnitude than in other mammalian cells. Thus, genetic manipulation can be done in DT40 cells in the presence of the appropriate selected markers, and then the modified chromosome can again be transferred to another cell line with an appropriate or convenient genetic background where gene expression studies can be performed (**Fig. 1**).

Thus, a typical experiment takes into account two steps that involve microcell fusion of particular human chromosomes that contain the genetic domain of interest and a previously integrated selected marker gene. At this point it is important to outline that for each microcell fusion event it is extremely important to verify the integrity of the transferred chromosomes, as during

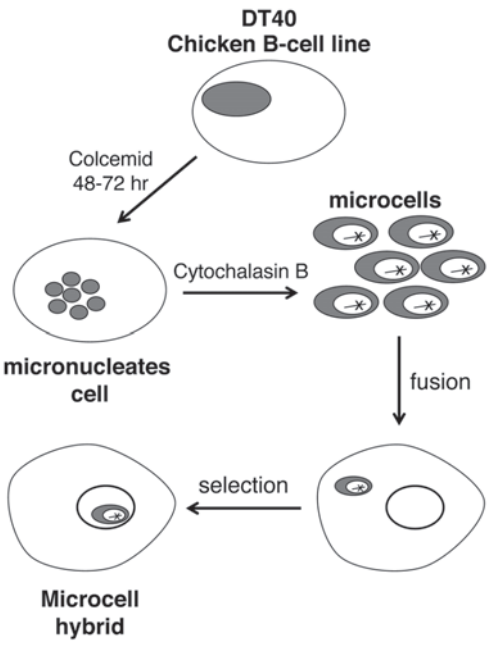

Fig. 1. Experimental procedure for the generation of microcell hybrids and modification of chromosomal DNA using homologous recombination in chicken B-cell tumor line DT40. The chromosome carrying the homologous recombination event is represented by an X. Homologous recombination events are selected and transferred into an appropriate mammalian cell line in which functional and chromatin structure can be done. Interestingly, the cell donor can come from mammalian cells or directly from the chicken DT40 cells. For example, the first donor cell could be from human origin, modified in DT40, and the modified human chromosome analyzed in a mouse cell line ***(57,59)***. At present, DT40 technology is well established and documented in http://swallow.gsf.de/dt40.html.

these procedures the loss of entire chromosomal regions had been observed with some frequency ***(57,58)***. To this end, systematic PCR analysis all along the chromosome with previously defined genetic markers, complemented with fluorescent *in situ* hybridization (FISH) are necessary to confirm chromosome integrity. Microcell fusion procedures coupled with homologous recombination techniques have been successfully applied to chromatin studies, particularly to the human and mouse β-globin loci ***(57,59)***.

In summary, microcell fusion coupled to the DT40 cell capacity to perform homologous recombination represents a clear alternative to knockout mice, and more broadly, the spectrum of cell types that can be analyzed.

4. Gene Transfer and Expression Problems

Gene transfer and expression are two experimental procedures that are confronted by a couple of problems. The gene transfer method and its associated efficiency is one of the key factors for a gene to reach the cell nucleus in the case of cell lines, or in the most difficult scenario, to access a particular cell type in a tissue. On the other hand, transgene expression represents a key component for experimental or therapeutic purposes. As mentioned previously, gene expression faces two obstacles: the first consists of a frequent variability in transgene expression levels, and the second, which is a less studied phenomenon, is the progressive extinction of expression or silencing of the transgene. In both cases, the main component responsible for such effects, also known as chromatin position effects, is chromatin structure ***(1,2)***.

4.1. Position Effects and Chromatin

There are two types of position effects: chromatin position effects caused by different integration sites and position effect variegation induced by a rearrangement and subsequent silencing of an active gene, frequently the result of its inactivation because of its proximity to heterochromatin ***(60–62)***. Briefly, position effect variegation has been defined as a stochastic and heritable silencing of gene expression. Originally described in *Drosophila*, it has been observed that the probability of gene expression is dependent on the strength of the regulatory elements of the transgene on the one hand, and on the other hand, on the chromatin environment surrounding the integration site ***(61,63)***. Such an effect is particularly accentuated when the integration of the transgene occurs close to heterochromatin where the silencing pressure is even stronger.

Chromatin position effects are basically understood to be the variability in gene expression as a

result of random insertion of each transgene in varied chromatin environments in the genome. Yet, how does chromatin structure affect transgene expression? In recent years, basic research and therapeutic strategies have inspired the study of the causes of transgene silencing over time in vivo. The majority of transgenes are subject to progressive silencing because of modifications in their chromatin organization, generating a highly repressive structure mediated by histone deacetylation and DNA methylation ***(1,2,64,65)***. At present it remains unclear which of these chromatin modifications occur first. In any case, initial observations show that in long-term cell culture, the histones of stably transfected genes are highly deacetylated and the CpGs located in their control elements are hypermethylated ***(1,2,65)***. Interestingly, hypermethylation of the promoter regions of tumor suppressor genes appears to be an alternative mechanism of gene silencing with direct consequences in the origins of tumorigenesis ***(66)***. As an indirect way to confirm transgene silencing by repressive chromatin conformation, histone deacetylase inhibitors like trichostatin A have been widely used, demonstrating a varied range of transgene expression reactivation ***(66,67)***. The 5-azacytidine or 5-aza-2'-deoxycytidine are DNA methylation inhibitors also capable of reactivating silenced transgenes ***(1,2,68)***. Interestingly, a connection between DNA methylation and histone deacetylation has been demonstrated, first by the interaction of methyl-CpG-binding proteins with methylated DNA, followed by the attraction of histone deacetylases by co-repressors ***(69)***. Based on such observations, when histone deacetylases and DNA methylation inhibitors are used simultaneously, the highest levels of gene expression reactivation are observed in the majority of cases ***(66,70)***. All of this evidence confirms the influence of chromatin structure on transgene expression. As a consequence, studies of chromatin remodeling mechanisms have been very useful in beginning to counteract the silencing effect of chromatin on integrated genes to reach sustained and homogeneous transgene expression.

4.2. Tissue-Specific Regulatory Elements

For any successful gene transfer and expression, the tissue or cell specificity should be carefully determined. In addition, the strength of the associated regulatory elements represents a complementary factor. Chromatin position effects have been overcome by the use of strong and dominant regulatory elements. Certain viral enhancer–promoter combinations are largely sufficient to overcome position effects, like the cytomegalovirus (CMV) promoter–enhancer elements (Rincón-Arano and Recillas-Targa, unpublished observations). Unfortunately, these elements usually possess a large spectrum of cell type activity making transgene expression too general. With the discovery of the LCR in the human β-globin locus, particular excitement was created with the possibility of overcoming chromatin position effects when those sequences were included in transgene vectors ***(71)***. The LCRs are defined as a group of DNase I hypersensitive sites located far upstream in the 5' noncoding region of the human β-globin domain ***(6)***. Two main functions for LCRs have been suggested: first, they have strong and specific enhancer activity, and second, they contribute to open chromatin structure at the domain level ***(6,7)***. The β-globin LCR provides strong erythroid-specific gene expression, and in its absence, the genes are now subject to strong chromatin position effects. When linked to a reporter gene, there is a copy number-dependent gene expression in erythroid cells, independent of the integration site ***(71)***. Therefore, the presence of the LCR has a positive and dominant effect on transgene expression independent of the chromatin integration context ***(6,7,72)***. Actually, a significant number of LCRs have been discovered, all of them being tissue specific ***(73)***. The principal restriction for the use of LCRs in recombinant gene expression is their tissue specificity. In some way this is not a problem, particularly when individual cell types need to be reached. Based on its properties, the LCR has been incorporated into transgene design in retroviral vectors and in the generation of transgenic mice ***(2,74,75)***.

4.3. Sustained Expression and Chromatin Insulators

A more recent and attractive alternative for transgene expression, once again based on basic research on chromatin, is the use of insulator or boundary elements *(2,5)*. Initially discovered in *Drosophila,* vertebrate insulators have emerged in different chromatin domains. At the present time the best-characterized insulator is the chicken β-globin insulator *(2,76)*. Insulators are functionally defined, based on two experimental properties: (1) they are able to interfere with enhancer–promoter communication exclusively when located between them, and (2) they have the capacity to protect a transgene, when located on each side of the vector, against chromatin position effects independently of the genomic integration site *(2,5,76)*. Usually, insulators are found to be constitutively hypersensitive to the action of nucleases, and in general they are neutral elements (i.e., they are not activators or repressors of transcriptional activity). All of these features, and particularly the ability to shield transgenes against chromatin position effects and progressive extinction of expression, show the real potential of insulators in transgenesis and gene therapy. A convincing example of the capacity of insulators to improve transgene expression comes from the chicken β-globin cHS4 insulator, originally defined as the 5' boundary of the locus *(76)*. Placing the cHS4 insulator on each side of a reporter vector contributes to a more constant level of gene expression, much more dependent on the control elements of the introduced transgene. In addition, the expression is maintained during long periods of time in stably transfected cell lines *(1,2,77)*. More recently, it has been clearly confirmed that the chicken cHS4 insulator recruits high levels of histone acetylation through the action of histone acetyl-transferases over the protected transgene, and at the same time it impedes DNA methylation of transgene control elements *(2,65,78)*. For the present discussion, we can say that insulators are becoming a realistic option to offset chromatin position effects in a majority of cell types and organisms without tissue-specific restrictions, as seen with LCRs. The cHS4 insulator has been successfully used to improve the number of transgenic mice founders and to obtain homogeneous gene expression in transgenic mice *(79,80)*. The same insulator has also been used in *Drosophila*, culture cell lines, and retrovirus vectors *(79–82)*. It is also worthwhile to mention that cHS4 and other insulators do not always protect a transgene against position effects. Therefore, much work remains to be done to understand insulator mechanisms and their spectrum of action. Thus, the use of chromatin insulators in combination with tissue-specific regulatory elements is getting closer to reality as a means of protecting against position effects with direct consequences in the expression patterns of transgenes and gene therapy vectors.

4.4. Alternatives to Chromatin Insulators

Not only have insulators been shown to be successful in shielding transgenes against position effects, matrix attachment regions (MARs) have also demonstrated their utility. The MARs are A+T-rich sequences biochemically defined based on their capacity to interact with the nuclear matrix *(83–85)*. The MAR sequences have been subject to certain skepticism in terms of their properties and contribution to gene regulation. They have frequently been proposed to drive chromatin loop formation, facilitating a topological configuration of a particular domain that indirectly favors gene activation. Consistent with this idea, the majority of MAR sequences have been found in noncoding regions flanking certain domains. The best example is the chicken lysozyme locus where two MAR sequences delimit the domain and show insulator properties, particularly by their capacity to improve gene expression in transgenic mice *(86,87)*. More recently, MAR sequences have been used in transgenic plants, dramatically improving gene expression *(88,89)*. At this point it is important to outline that not all MAR sequences are able to improve transgene expression, and MARs from distinct loci do not give consistent results.

5. Retroviral Infection and Gene Therapy

During the past 15 yr gene therapy has become an important and useful strategy with the potential to correct diverse diseases *(2,90)*. Gene or genetic therapy faces one of the most exciting times. Novel strategies for gene transfer take into account the use of diverse types of viral vectors. Viral vectors demonstrated their ability to stably transform cultured cells and even to produce tumors in animal models that represent a useful approach for probing the mechanisms of oncogenesis. Viral vectors have proved to be more efficient than other gene transfer strategies. Unfortunately, viral vectors are also subject to position effects and to their own viral structural and regulatory elements, which causes progressive gene silencing, thereby preventing sustained transgene expression *(74)*. Another consideration is that viral gene expression faces an additional problem in the ability of the viral particle to reach the desired target cell type *(91)*. Thus, originally defined to contribute to the treatment or prevention of diseases, gene transfer has now found serious limitations, in particular with the risks of "insertional mutagenesis" of viral and nonviral vectors with devastating consequences *(90)*.

As stated earlier, one of the critical aspects of introducing a transgene into the genome is the variability or even total lack of gene expression. This is mainly attributed to the chromatin environment at the genomic insertion site. But this is no the only difficulty, in the case of viral vectors, several components can jeopardize their expression such as enhancers, but mainly silencers, particular class of repetitive sequences (LTRs), and a high concentration of CpG-dinucleotides (target sequence motifs for DNA methylation). In general terms, it is now accepted that the regulatory elements capable of counteracting chromosomal position effects may be divided in two groups: (1) the one with dominant positive regulatory elements that imposes an active chromatin configuration and (2) those with barrier or insulating capacity that can physically block the passage of chromatin structures and signals (either positive or negative) from the surrounding chromatin. Therefore, these need to be taken into consideration when we want to design and use a viral vector.

5.1. Viral Vectors and Chromatin Insulators

Distinct types of viruses have been engineered to become highly specific and efficient for gene delivery to particular cell types or tissues *(2,90,92)*. Several types of viruses, including retroviruses, adenoviruses, adeno-associated viruses (AAV), herpes simplex viruses, and more recently, lentiviruses have been modified in the laboratory for their potential use in gene transfer and therapy applications. Depending on the cell type, tissue, and reporter gene used, complementary modifications of viral vectors are needed and one attractive option is the incorporation of insulator sequences, with the aim to ameliorate transgene expression. Complementary modification can be considered like codon usage optimization and modifications of regulatory elements (like viral silencers), as well as elimination of putative mammalian RNA processing signals or CpG-dinucleotides *(2)*.

In the case of retroviral vectors, there are retroviral regulatory elements that can induce epigenetic silencing with a progressive increase in DNA methylation and methyl-CpG-binding protein recruitment, along with histone repressive marks like histone deacetylation and methylation *(66,93,94)*. As a means of combating these negative viral components, it has been demonstrated that the chicken β-globin cHS4 insulator is able to block retroviral silencing in a murine stem cell virus (MSCV) vector in transgenic mice *(95)*. Interestingly, the results obtained with this MSCV retrovirus are in agreement with the data published previously in chicken stably transfected cell lines *(1,2,77)*. In another study with retroviral vectors, Rivella and collaborators *(96)* demonstrated that viral transgene expression generated in mouse erythroleukemia cells in the absence of drug selection is improved by the presence of insulators, which can reduce the *de novo* methylation originating at the 5' LTR of the retroviral vector. Another example is the use of retrovirus in com-

bination with the chicken cHS4 insulator and the incorporation of the α-globin LCR (αHS-40) to drive expression of the human $^{A}\gamma$-globin gene in mouse bone marrow transduction and transplantation assays ***(97)***. In these studies, there was a clear effect ranging from 2 to 5% expression for the noninsulated oncoretrovirus vector to 49% for the insulated vector ***(97)***. Such an improvement represents a reasonable increase in transgene expression that may allow us to reach therapeutic expression levels in future hemoglobinopathy protocols ***(98,99)***. Adeno-associated virus vectors have also been tested for their improved capacity for gene expression in the presence of cHS4 insulator ***(2)***.

In recent years, a viral alternative has emerged with lentivirus vectors based on their genetic stability, reduced inflammatory and immune response ***(90)***, but they are still subject to chromosomal position effects. There is an interesting investigation in which scaffold attachment sequences, in combination with the chicken β-globin cHS4 insulator, but not with the cHS4 insulator alone, are not able to quantitatively improve transgene expression, but instead they qualitatively ameliorate green fluorescent protein expression. These results support the notion that the lentivirus transgene is not increasing the number of expressing cells, but in contrast is counteracting chromosomal position effects, and in this example probably variegation ***(100)***. A novel aspect emerging from these series of studies is that chromatin elements being either a matrix/scaffold or an insulator sequence, in some cases they are sufficient for homogeneous and sustained gene expression levels in viral vectors, shows that a combination of these elements could contribute more effectively, with each element having its individual properties for improvement of transgene expression. Thus, the incorporation of two distinct protective sequences in viral vectors may also contribute to the stability, integrity, and titer numbers of lentiviruses. This is because two identical sequence shielding the vectors maybe interpreted by the cell as repetitive elements favoring undesirable recombination events. Most interestingly, in a recent publication a lentivirus vector carrying the human β-globin expression cassette with the incorporation of chromatin cHS4 insulator restore normal levels of human β-globin expression in erythroid cells and counteracting previously observed chromosomal position effects ***(98)***.

In summary, chromatin insulators or boundary elements have emerged as favorable candidates to improve transgene expression. In fact, the main goal is to establish, or at least attempt to estimate, appropriate gene transfer levels of the therapeutic gene, and of course, to reach adequate expression levels to achieve curative or corrective effects. Consequently, it is clear that several considerations are needed for viral vectors optimal design and development: (1) knowledge of internal regulatory and structural viral elements like enhancers, silencer, and LTRs; (2) based on such a distribution, the location where the insulators sequences will be incorporated is crucial; (3) the choice of transgene regulatory elements (strength and tissue specificity); (4) avoidance of elements detrimental to transgene expression, such as CpG-dinucleotides to minimize aberrant DNA methylation; (5) the size and combination of insulators to be used to favor elevated titer production and improve virus stability; and (6) presence of both insulator activities, enhancer or silencer blocking and protection against chromosomal position effects to ensure maximal action.

In conclusion, it is fair to mention that despite some limitations and the fact that we do not fully understand how they function, insulator elements will probably contribute to the next generation of gene therapy viral vectors.

6. Conclusions and Prospects

Gene transfer and transgene expression represents one of the most frequently used approaches to address different scientific and practical questions. As mentioned the particular method used to transfer DNA molecules, chromatin environment, and specific regulatory elements associated with a transgene is critical in obtaining convenient patterns of expression. Alternative strategies are recommended to confirm the obtained results. In any

case, a growing number of laboratories are concentrating their efforts on developing strategies for a more uniform and sustained level of transgene expression. The MAR and insulator sequences require particular attention in the near future. At this point, there is no question that nuclear organization and dynamics combined with chromatin structure and remodeling activities need to be taken into account to establish better gene transfer protocols in the future.

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