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Review

Transgenesis for the study and the control of lactation

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Abstract — The study and the control of milk synthesis are required to decipher the mechanisms of gene expression, to improve milk production, to modify milk composition, to induce a resistance to diseases in the mammary gland and to produce recombinant proteins of pharmaceutical interest. Transgenesis has become a mandatory tool to reach these goals. The use of transgenesis is still limited by the difficulty of adding foreign genes in farm animals and mainly by replacing genes by homologous recombination. Transgene expression is also often ill-controlled. The present paper summarizes the current progress in this field with a particular emphasis on expression vectors for transgenes.

lactation / transgenesis / expression vectors

Résumé — **La transgénèse pour l'étude et le contrôle de la lactation.** L'étude et le contrôle de la synthèse du lait sont nécessaires pour décrypter les mécanismes de l'expression de gènes, pour améliorer la production de lait, pour modifier la composition du lait, pour induire une résistance de la glande mammaire contre des maladies infectieuses et pour produire des protéines recombinantes d'intérêt pharmaceutique. La transgénèse est devenue un outil indispensable pour mener à bien ces projets. La mise en œuvre de la transgénèse est encore limitée par la difficulté d'ajouter des gènes étrangers chez les animaux d'élevage et surtout de remplacer des gènes par recombinaison homologue. L'expression des transgènes est par ailleurs souvent mal contrôlée. Cet article se propose de faire le point dans ce domaine en développant plus particulièrement la mise au point de vecteurs d'expression pour les transgènes.

lactation / transgénèse / vecteurs d'expression

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1. INTRODUCTION

Milk is a major source of food in developed countries. Improving milk production and composition has been a constant effort for centuries. Genetic selection, nutrition and the struggle against diseases have contributed to the success of the milk industry.

Molecular genetics and genetic engineering, including transgenesis, now offer additional possibilities to improving milk production, optimising milk composition, protecting the mammary gland against infectious diseases and producing pharmaceuticals in milk. These tools are also essential in order to understand the mechanisms involved in milk synthesis and secretion.

Transgenesis gives the possibility of adding foreign genes to be expressed in the mammary gland or elsewhere into the body of animals. Gene targeting by homologous recombination leading to allele replacement is also theoretically possible. The targeted expression of transgenes in the mammary gland has been achieved successfully for more than a decade using the regulatory region from several milk protein genes. The aim is often to target the expression of a foreign gene in the mammary gland at a high rate. This is obviously the case when a foreign milk protein to be used as a source of food or pharmaceuticals is to be produced in milk. Potent and reliable vectors are required to reach this goal. Despite an impressive success, the expression rate of many transgenes remains not fully predictable. Numerous works have been undertaken to improve transgene expression in the mammary gland.

In addition, a certain number of projects are in course to improve milk production by farm animals for human and animal consumption and to produce high value foreign proteins in milk.

These different experimental approaches of lactation are briefly reviewed in the present paper.

2. GENE TRANSFER IN FARM ANIMALS

2.1. Gene addition

A foreign DNA fragment introduced into a cell or an embryo, can become stably integrated into the genome. This integration process occurs randomly leading in some cases to the disruption of a host gene and more frequently to the silencing of the transgene.

Microinjection of foreign DNA into the pronucleus of an embryo is the method of choice to generate transgenic mammals. Yet, this technique is poorly efficient in ruminants. Optimisation of the method has been reached by preparing embryos by in vitro maturation of oocytes collected in the slaughterhouse, in vitro fertilization and in vitro development of the embryos after DNA microinjection until the blastocyst stage. This method does not imply the use of embryo donors and it considerably reduces the number of recipients [23].

The implementation of the cloning technique by nuclear transfer has considerably facilitated transgenesis in ruminants. Foreign genes are stably transfected into somatic cells which are used to generate transgenic clones. This approach offers several advantages. The yield of transgenesis is improved, even if animal cloning is laborious. The integrated gene can be examined before proceeding to nuclear transfer. This method is without contest more attractive than microinjection for sheep [35], goats [19, 32] and cows [2, 43].

2.2. Gene replacement

Targeted gene transfer leading to gene replacement has allowed the specific inactivation of about 5000 genes in mice. The technique defined more than a decade ago consists in replacing a gene by homologous recombination in embryonic stem cells (ES), which have the capacity to generate chimeric

animals after having been introduced by micro-manipulation into early embryos [5].

This method has failed in all species other than mice. This is clearly due to the fact that no equivalent of the mouse ES cells has been found in other species (although recent studies suggest that this might be the case in chickens and medaks).

The cloning technique used to add genes to farm animals can theoretically be implemented as well to replace genes by homologous recombination. This goal has been reached in sheep [25, 31] and pigs [24].

However, gene replacement by cloning is a difficult task [9]. The major problem comes from the cloning step and not from gene targeting. The selection of the cells in which gene replacement occurs necessitates several weeks. At the end of the selection, many cells have exhausted their theoretical capacity to multiply. More importantly, the physiological state of the cell is altered during culture leading to a quite reduced capacity of the cells to generate viable cloned animals.

Improvement of the cloning technique is thus required before gene replacement may become a routine in farm animals. It seems that the success met by PPL Therapeutics to generate sheep and pigs in which genes have been replaced was the result of a large number of trials, a strategy compatible with medical or pharmaceutical projects but not to improving animal production.

2.3. Gene targeting

The introduction of a foreign gene in a chosen site of a genome offers several theoretical advantages. The site may have been chosen for its capacity to favor transgene expression. Even if the expression of the transgene in a given integration site is subjected to a position effect of the chromatin, this artefact is expected to be constant. This reduces the number of transgenic lines from which no relevant information can be obtained. The integration site may have been chosen in an empirical manner after the observation that a transgene at this position in the genome was well expressed. Alternatively, a foreign DNA known to contain elements favoring transgene expression (such as insulators) may have been previously introduced in the genome by random or targeted integration using conventional homologous recombination.

The targeted integration of the foreign DNA may then be achieved by the natural homologous recombination mechanism. It is well-known that this event is very rare and in practise this approach is very laborious and possible essentially in mice.

The integration of foreign DNA is known to occur during DNA replication using a repair mechanism. This mechanism is highly stimulated by DNA cleavage. DNA cleavage may be induced at a chosen site. This is achieved by previously introducing a I-Sce 1 restriction site by gene addition or replacement. The cointroduction of a recombination vector and a circular plasmid expressing the yeast meganuclease I-Sce 1 gene markedly enhances the frequency of recombination at the I-Sce 1 genome site without damaging the host genome which has little chance to contain an endogenous I-Sce 1 [7].

LoxP or FRT sites which recombine with a high efficiency in the presence of specific Cre and Flp recombinases respectively may be added to the site of integration. The cointroduction in a cell of foreign DNA containing a LoxP or FRT site and of a plasmid expressing the corresponding recombinase gene leads to a targeted integration at the chosen site of the genome [8].

The yield of this method is low, due to a large extent to the fact that the recombinase has greater chance of eliminating the integrated DNA fragment containing LoxP than of integrating it. The action mass rule becomes in favor of the integration when two different LoxP sequences, which cannot recombine, are used (one in the vector and

the other in the integration site) [1]. This method is, however, poorly efficient.

Another possibility consists in inducing recombination in one cell embryo with the Cre recombinase brought by the sperm. This method named TAMERE (targeted meiotic recombination) has proven to be efficient in recombining homeotic genes [13].

The use of two independent and non-compatible LoxP sites instead of one in the vectors and one in the site of integration appears presently to be the most attractive way of using the LoxP-Cre system to integrate a foreign gene in a targeted region of the genome [3, 10].

All these methods, leading to the targeted integration of foreign genes, have been used in cultured cells. They imply a selection of the cells in which the desired integration occurred and the use of these cells to generate an embryo either by chimerism with ES cells or by nuclear transfer with somatic cells. This presently limitates the use of these techniques in farm animals. It is not known if the direct microinjection into the embryo might induce targeted integration at an acceptable rate.

3. DESIGN OF A VECTOR FOR TRANSGENE EXPRESSION

The first gene transfer in mice two decades ago revealed that gene constructs which are quite active in cultured cells are often silent in transgenic animals. A certain number of rules emerged to explain this phenomenon.

A transgene is poorly expressed when it is integrated into a non active region of the genome (centromere, telomere, intergenic regions). This position effect may as well allow the expression of a transgene in cell types in which the promoter used is not expected to work. This is attributed to the long distance action of enhancers which belong to endogenous genes. However, transgene silencing is more frequent and more intense than their leaky expression. This inclines one to think that the transgene itself might induce its own extinction.

A transgene is usually much less expressed when its transcribed region is a cDNA rather than a genomic fragment. This indicates that signals for mRNA processing and transfer to the cytoplasm are present in introns. Other signals for mRNA translation and stability are often found in 5' and 3'UTR. A transgene is also frequently silent when it is integrated into arrays containing many copies. Another phenomenon has also been identified. A transgene is extinguished to a variable degree when it contains numerous CpG motives, a situation encountered in invertebrate and mainly prokaryotic genes.

The extinguished transgenes are methylated and maintained in heterochromatin. This silencing mechanism seems similar to that involved in the inactivation of integrated transposons and retroviral genomes.

To be expressed, a gene must contain at least a promoter-enhancer region, a coding sequence and a transcription termination. Most of the transgenes contain only these minimum elements plus one or several introns. This is clearly insufficient in many cases to obtain a satisfactory expression of the transgene. Besides, data in the literature report that long genomic DNA fragments (80–500 kb) are able to express the genes they contain in transgenic animals in a quite appropriate manner. About 30 transgenes have thus been expressed in all the lines of animals, with a strict tissue specific pattern and as a function of copy number.

For the sake of simplicity, a gene can be arbitrarily divided into three functional regions: the distal region containing insulators, the promoter-enhancer region and the transcribed region. Although these elements cooperate, it is possible to study the three regions separately.

3.1. The promoter-enhancer region

The promoter corresponds to the region in which the preinitiation transcription complex is formed. It is contained in no more than 150 bases located immediately upstream of the cap site. The enhancers are spread over 10 kb stretches of DNA or sometimes more. Empirically it is possible to define the region containing the enhancer and the promoter even if they are not known in detail. This is sufficient to define the genomic DNA region to be added in a construct to obtain cell specific transgene expression.

Enhancers are more and more considered as acting by increasing the frequency of promoter activity rather than by amplifying the promoter action. The transcription of a stimulated gene occurs only part of the time, leading to a varied expression. Enhancers lengthen the periods during which a gene is expressed, leading to a global enhanced expression.

A promoter-enhancer block may be boosted by adding enhancers. Experiments carried out in our laboratory showed that six copies in tandem of the major enhancer regions from the rabbit αS1 casein gene are exquisitely induced by prolactin and the extra cellular matrix probably through the action of Stat5 and C/EBPβ [18, 28]. These six copies of the enhancer are capable of stimulating the expression of a reporter gene driven by the herpes simplex thymidine kinase promoter and the 6.3 kb promoter of the rabbit WAP (whey acidic protein) gene in the HC11 mammary cell line. This stimulatory effect is not significant in transgenic mice [28, 29] and the polymerized enhancers do not prevent the WAP gene promoter from being sensitive to the position effect. These observations suggest that the 6.3 kb region used probably contains all the promoter-enhancer elements but that other regulators have to be found in far upstream regions.

3.2. The insulator region

The study of various loci have revealed that genes or gene clusters are separated from each other by boarder regions which have different functions. Some of these regions named LCR (locus control regions) contain enhancers and insulators. The enhancers control the expression of the genes present in the locus. It is not clear how these enhancers cooperate with the more proximal enhancers.

A fascinating idea was recently proposed. The LCR of the β-globin locus, which is the most thoroughly studied, contains several enhancer regions, which are mandatory for the expression of the globin genes. RNA polymerase II has been found in the LCR regions probably via the factors bound to the enhancers. RNA polymerase II is not associated to a promoter since the LCR does not contain any promoter. The RNA polymerase II stored at the LCR level might be transferred to the gene of the locus, which becomes open by the appropriate transcription factors [17]. The LCRs also contain an insulator, which has been more precisely located in the chicken β-globin locus. The 5'HS4 insulator has the capacity to inhibit the stimulatory effect of the $5'HS_2$ enhancer from the β-globin locus on the β-globin promoter in myeloid cells. The insulator thus prevents the action of the enhancers of the gene located immediately upstream of the β-globin locus. The insulator also seems to be able to prevent the spreading of heterochromatin and thus the extinction of a locus [4].

This property of the 5'HS4 insulator urged us to use it for protecting transgenes from extinction. This was the case. The 5'HS4 region was added in duplicate upstream of the transgenes driven by the EF1-α gene promoter which is active in all cell types [38] and by the WAP gene promoter [33]. In both cases the transgenes bearing the 5'HS4 insulator were expressed in all the lines of transgenic animals whereas the same constructs

devoid of the insulator were expressed at a reduced rate or not at all. Hence, the 5'HS4 insulator has a broad spectrum of activity but with only a limited effect. Indeed, the expression of the transgenes remained independent of the copy number.

Large DNA fragments containing the α-lactalbumin [12, 37] or the WAP gene [34] were used to generate transgenic mice. In these three cases, the transgenes were expressed strictly in the mammary gland of the lactating animals, at a high level and as a function of copy number. A foreign gene introduced in the human α-lactalbumin genome fragment was highly expressed in transgenic animals. These genomic regions can therefore be used as such to express foreign genes, in an appropriate manner.

Experiments are in progress to identify the insulators present in these long DNA genomic fragments. This will give the possibility to study their mechanism of action and to use shorter genomic fragments to express transgenes in a reliable way.

3.3. The transcribed region

The transcribed region of eukaryote genes contains a number of signals which were still unknown a few years ago. The 5'UTR (untranslated region) often contains signals which control translation [14]. The 3'UTR has sequences which control mRNA stability and targeting in cell compartments [26].

Binding sites for transcription factors are often found in 5'UTR but also in introns. They may contribute to maintaining chromatin in an open configuration. In a recent study it was shown that introns enhance the elongation rate in RNA synthesis [11]. This effect results from an interaction between the phosphorylated carboxyl-terminal domain of RNA polymerase II and a splicing factor. A reciprocal synergism seems to occur as well. The RNA polymerase II favors the action of splicing factors.

Intron elimination implies the formation of nucleoprotein complexes which bind to the splicing region. The mature mRNA keeps part of the complex which is required for the transfer of the mRNA from the nucleus to the cytoplasm [21].

The presence of a termination codon located at more than 50 nucleotides upstream of a donor splicing site triggers a mechanism named NMD (non-sense mediated decay) which induces the destruction of the mRNA. Natural mRNAs do not have a termination codon in a position which can induce NMD. This surveillance mechanism which acts both in the nucleus (where mRNAs are translated) and in the cytoplasm, eliminates the mutated mRNAs coding for truncated proteins [42].

All these mechanisms have to be taken into account to construct chimeric genes. All these points are discussed in more detail in a chapter of a book in press [15]. To the best of our knowledge, cassettes capable of expressing transgenes harboring various cDNAs in a reliable manner have not yet been designed.

4. TRANSGENESIS TO IMPROVE LACTATION

The content or composition of milk from domestic ruminants might be optimised by transgenesis [30] and such experiments are under way.

A reduction in lactose content has been obtained in the milk of laboratory animals [40]. The expression of lactase in milk seems to be a promising approach. Some of these protocols might be extended to the cow and may reduce intolerance to lactose for many consumers.

Phenylalanine-free α-lactalbumin has been produced in large amounts in transgenic cow milk (PPL Therapeutics, unpublished data). This particular protein might be a source of amino acids for patients suffering from phenylketonurea.

Transgenic pigs expressing bovine α-lactalbumin in their milk nourish their piglets more efficiently. The effect of other factors, such as IGF1 and lactoferin are also under study [41].

Recombinant antibodies secreted in mouse milk have been able to prevent infection by the corona virus [22, 36].

Two antigens of rotavirus have been prepared in mouse milk. These proteins showed the capacity to induce antibody formation after injection into the mouse (unpublished data).

Mice expressing lysostaphin, a procaryotic protein having antibacterial activity, have been obtained. The mammary gland of these animals are resistant to experimental infection by *Staphylococcus aureus* [20]. Mice expressing the lysozyme gene are also under study [27].

The preparation of recombinant protein at an industrial scale in milk of farm animals was undertaken more than a decade ago [16]. Several proteins, namely monoclonal antibodies, are under clinical examination. The human α-glucosidase prepared from transgenic rabbit milk generated in our laboratory improved the health of babies suffering from the Pompe disease [39]. After months, these babies are still alive. This would not have been the case for some of them if they had not received the treatment.

Hepatic steatosis is a common problem of the lactating cow during the first part of lactation. It is admitted that this results from an excess of lipid accumulation in the liver. The laboratory animal models suggest that an overexpression of apolipoprotein E and MTP genes in the liver might release lipids into the blood circulation [6]. Steatosis is believed to alter the health of lactating cows leading to a reduction in milk production, an increased fragility towards diseases and a higher frequency of mammary infection. Lactation might thus be indirectly improved by the expression of two transgenes in the liver.

Transgenesis can also be a powerful tool to elucidate the physiological aspects of lactation in farm animals.

5. CONCLUSION AND PERSPECTIVE

Transgenesis has brought invaluable information on the mechanisms of mammary gland growth and milk synthesis. The applications of this technique are still not numerous. The production of pharmaceuticals in milk is reaching maturity and the first protein should be on the market soon. It may be followed by many others.

The modification of milk composition is a more complex technical problem in some cases, namely when alleles have to be replaced by homologous recombination. It is interesting that pig milk was improved in this way. The techniques available need improvement but they may be used in their present state for a certain number of projects. Even optimized, these techniques will remain relatively laborious and costly. Their use will therefore be justified only for projects leading to a sufficient improvement of milk production and when no other approach is successful.

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