Variation in microsatellite sequences between tilapia belonging to the *Oreochromis* genus

Mario P Estrada, Orelvis Portal, José de la Fuente

Polymorphic animal microsatellites have proved valuable genetic markers. In this report, conditions were established to examine the variability of 6 tilapia *(Oreochromis niloticus)* microsatellite loci between tilapia belonging to two species *(O. niloticus* and *O. aureus)* and one transgenic *O. hornorum* hybrid (F70) of the genera *Oreochromis*. The heterozigosity of the microsatellites was determined and the paternity index and power of exclusion calculated, showing that tilapia microsatellites are powerful tools both in regard to gene mapping, population genetic analysis and for individual identification.

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Population structure of the shrimp *Penaeus notialis* assessed using allozymes and mitochondrial DNA

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In the present study we investigate the genetic variation among populations of the shrimp *Penaeus notialis*, the most abundant penaeid specie around Cuba. In this regard, samples of shrimps from five localities in the south central platform of the island (Ana María Gulf) were analyzed using nine polymorphic allozyme loci and PCR-RFLP of a segment of 2072 pb of the mtDNA comprised between the COI and COIII genes.

Of the 25 allozyme loci studied 9 resulted polymorphic and presented no deviation from Hardy-Weingber proportions. Among them three loci contributed significantly to the genetic variation observed Gdh, Est-3 and Fos-2. In contrast to mtDNA at the Ana María Gulf, the level of genetic differentiation from allozyme was significant among some localities. Manatí and Florida were different compared to the rest and among them and a limited genetic flow was evidenced. However, homogeneity of the mtDNA suggested that differentiation al allozyme loci should correspond rather than to recent events to a historical isolation of this subpopulations. The current pattern in the region and some other biological parameters support the results. The genetic distinction of mtDNA among subpopulations from Batabanó Gulf and Ana María Gulf indicates that this marker will be useful to study the genetic structure of *P. notialis* along the Cuban platform.

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MAMMARY GLAND TRANSGENESIS: TODAY AND TOMORROW

Fidel Ovidio Castro

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The field of transgenesis in mammals is moving quite fast. In 1983, there were only a few reports about transgenic animals in the main stream of scientific literature. By the end of 1998, in less than 15 years, we have to face the fact that somatic nuclear transfer transgenic livestock has been produced. Needless to mention the establishment of gene replacement in murine embryogenic stem cells, the production of high-value pharmaceutical proteins in the milk of transgenic mammals or pigs for the xenotrasplantation of human organs, just to mention some of the more quickly developing areas of research and investment.

The annual meeting Biotecnología Habana '98 is a good witness of these developments. In our previous edition devoted to transgenesis in mammals during Biotecnología Habana '95 there were important contributions from relevant scientists on the regulation of milk protein gene expression, from the basic science to biotechnology. A lot of effort was then centered on the use of transgenic bioreactors for the production of human therapeutics in the milk.

In this edition the field moved further, as it can be noticed in the following examples:

- Knock-out mice lacking important regulatory elements for milk gene transcription have been produced and characterized.
- Gene ablation of the crucial prolactin receptor was achieved *in vivo* in mice.
- Evidences are on hands about the existence of a casein gene locus controlling region at least in the bovine.
- The role of internal ribosome entry sites (IRES) in the expression vectors has been deeply questioned and instead new data appeared about the role of these sequences in the rescue of translation stimulators (RTS).
- The post-translational machinery of the mammary gland has been challenged with new hard tasks such as the expression of vitamin K-dependent gamma-carboxilated proteins in pigs and recombinant antibodies in rabbits.
- New emerging technologies have gained a leading role in the generation transgenic animals express-

ing in mammary gland, among those are of outstanding interest:

1 the expression of fused, inactive precursor proteins for peptides of known potent biological activity
2 the use of yeast artificial chromosomes (YAC) for the cloning and expression of megafragments of regulatory and coding sequences of milk protein genes
3 the use of somatic cloning techniques for the generation of transgenic ruminants and the first steps toward the establishment of this technology in rabbits

Undoubtedly, the irruption of the gene knockout technology with its wide applications to all the fields of current molecular biology have had a tremendous impact on the mammary gland transgenesis as well. Jeff Rosen and colleagues inactivated the endogenous C/EBP genes in their a and b variants. These studies revealed the role of C/EBPb in ductal morphogenesis, lobuloalveolar and functional differentiation, leading to drastic lowering of casein milk gene expression. In line with this, Lothar Hennighausen's group knocked-down the Stat5a transcription factor gene. This led to a phenotype very similar to that described for the C/EBPb knock-out mice. Paul Kelly and co-workers in Paris disrupted the gene of the pivotal prolactin receptor leading, as it was expected, to a failure in mammary gland development and lack of lactation. Interestingly, the heterozygous females were able to nurse after the second parturition, probably indicating that the animal can somehow compensate a partial (one-allele) ablation of the prolactin receptor. Taken altogether, the aforementioned experiments highlighted the role of important steps in the complex pathway of mammary gland gene expression and could shine a light over breakthrough experiments aimed to improve expression vectors for transgenesis and also cancer research.

However, mammary gland transgenesis is still in its childhood. The poor knowledge we still have about the mechanisms governing milk gene expression together with the current limitations of transgenic technology make it difficult to routinely produce high-value pharmaceutical or nutraceutical products in the milk of transgenic animals. Undoubtedly, one of the most important problems still to be solved is the design of appropriate expression vectors for mammary gland gene expression. The search for milk locus controlling regions (LCR) continues, and at least with the bovine and murine casein gene locus there seems to be optimistic winds blowing from the results of the group at Baylor College in Houston. Scientists at Baylor found evidences for a LCR in the casein locus, however, transgenic experiments in which the putative regulatory sequences responsible for LCR activity are included need still to be done as conclusive proof.

The evaluation of the post-translational (PT) processing capacity of the mammary gland is crucial for research on transgenic livestock bioreactor feasibility. Experiments with transgenic pigs expressing recombinant proteins pointed out that a trial production of each recombinant protein may be required in order to deduce the capacity for PT-modification with any degree of certainty for a given complex protein.

The pioneer work of the British teams at Roslin and PPL in Scotland started to pave the way for the real settlement of the somatic cloning technology. Their work demonstrated for the first time that genetic manipulation of somatic cells does not compromise their totipotency. The implications of this are enormous; all the animals will bear the transgene in all their cells, specific DNA sequences can be added or removed at will, instant flocks or herds of animals can be made and subsequently all the transgenic animals required for the production of a given protein in their milk can be produced at the same time, from the same animal, in just one generation, from frozen cells. Noteworthy, scientists have termed these cells "protoanimals". There is no doubt that the revolution in science caused by Dolly will have a notorious impact on our future in all means, but especially in transgenesis.

It is clear that although the field of mammary gland transgenesis is mined with big difficulties, there have been important achievements in the last years. Several of them were accomplished during the window between the two editions of Biotecnología Habana devoted to mammary gland transgenesis. Among those, I would like to highlight the following:

- establishment of high expressing flocks of sheep producing tens of grams per liter of therapeutic proteins
- beginning of clinical trials for cystic fibrosis (United Kingdom, USA) and congenital emphysema (United Kingdom) from sheep-derived transgenic proteins
- transgenic cattle expressing human α-lactoalbumin in their milk at high levels
- production of transgenic factor IX and protein C in pig's milk
- replacement of the endogenous mouse α-lactoalbumin milk gene with the human counterpart, and high expression of it in gene- replaced mice
- cloning and expression of human a-lactalbumin megafragment gene using YAC technology in transgenic rats
- birth of Dolly the sheep
- birth of transgenic and cloned sheep and cattle from somatic cells
- birth of cloned mice from somatic cells

Where do we go from here?

The next edition of our serial meeting; already in the next century will probably bear witness to transgenic production of proteins in the milk of cloned ruminant species, the wide use of somatic cloning technology in mice, rabbits and pigs. New pharmaceutical proteins will be under examination in clinical trials, while the first patients will probably buy prescribed milk-derived transgenic drugs, right next corner in the pharmacy of the neighborhood. But let's try to figure out which will be the challenges that await us in the way of mammary gland transgenesis in our next meeting. This is always a risky and not a grateful task, but it is worth trying.

In the field of mammary gland gene expression:

- new gene replacement experiments of transcription factors specific for mammary gland expression will be performed and relevant information will be collected
- of outstanding interest will be the introduction of the conditional knock-out technology in the mammary gland. By these means, one should be able to study the role of a given gene that is abruptly eliminated from the genome during different stages of mammary development and nursing. Especially interesting will be the conditional knock-out of the Stat5a.b genes as well as the C/EBP genes
- the knock-out of the WAP gene is eagerly awaited as the role of the protein in mammary gland development seems to be crucial and to certain degree it is still unknown
- means for external inducible control of transgene expression in the mammary gland will be available on the basis of the tetracycline regulatory system
- new promoters of milk protein genes will be cloned, characterized and used in transgenic studies
- locus controlling regions from the casein genes will be already characterized
- independent transcription units capable of tissue-specific, copy number-dependent and site-independent integration will be available for transgenic expression
- increased molecular resources will be available from the gene pool of wildlife species
- while we still await for embryonic stem cells from livestock, ribozymes might be an alternative for targeting and destroying specific mRNAs in the mammary gland. This is especially true for genes where extremely low protein levels would be acceptable
- in the technology itself
- better protocols for *in vivo* transomatic transient expression of transgenes will be designed and tested. One can foresee that organic polycations will be the leading vehicles for this kind of gene transfer
- embryonic stem cells amenable for genetic manipulations might be available from farm animals
- somatic nuclear cloning will become more and more widely used in ruminants and also in "hard to clone" species such as rabbits and pigs
- factor(s) responsible for nuclear reprogramming in somatic nuclear transfer experiments might be identified and cloned
- the use of male germ cells as vehicles for gene transfer definitively deserves more research efforts
- YAC technology will be extended to farm animals for the expression of recombinant proteins in their milk

These and many other applications will be the milestones of the mammary gland transgenesis in the next century.

Regulation of milk protein gene expression: insigths from transgenic and knock-out mice

JM Rosen, S Lindsey-Wyszomierski, T Seagroves

Previous studies have established that composite response elements (CoRE) containing multiple binding sites for several transcription factors mediate the hormonal and developmental regulation of milk protein gene expression

In the casein promoters these include binding sites for signal transducers and activators of transcription (Stat)5, Yin Yang (YY)-1, CCAAT/enhancer binding protein (C/EBP) and the glucocorticoid receptor (GR). In the absence of the lactogenic hormones, insulin, hydrocortisone and prolactin, the casein promoter is repressed by the interaction of LIP (a dominant negative form of C/EBPb) and YY-1, which may recruit co-repressors with histone deacetylase activity. In the presence of lactogenic hormones, transcriptional activation is conferred by a pleiotropic mechanism involving cooperation of the transactivation domains of STAT5 and GR and enhancement of STAT5 DNA binding by GR. GR enhances STAT5 activation by prolonging its tyrosine phosphorylation, an interesting example of how transcription factors in CoREs may be able to modulate each other's activities. GR has also been shown to interact directly with C/EBPb, so protein:protein interactions are likely to stabilize the binding of each individual transcription factor to its response element thereby creating a stable activation complex.

The functional importance of individual C/EBPs for mammary gland development and differentiation has been demonstrated in mice which carry targeted deletions of C/EBPb and C/EBPa.

These studies revealed that C/EBPb is required for ductal morphogenesis, lobuloalveolar development and functional differentiation. In primary mammary epithelial cultures derived from the C/EBP KO mice, expression of b-casein was inhibited 85-100% while whey acidic protein (WAP) was undetectable. Finally, STAT5, GR and C/EBPb have all been shown to interact with p300/CBP, coactivators with histone acetyltransferase activity, so recruitment of p300 or CBP is likely to be an essential component of this transcription complex. (Supported by grant CA16303 from the National Institutes of Health).

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From the molecular biology of prolactin and its receptor to the lessons learned from knockout mice models

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Prolactin (PRL) along with growth hormone (GH) and placental lactogen (PL) form a family of hor-

mones which probably evolved from the duplication of an ancestral gene. PRL and GH are mainly synthesized by the pituitary in all vertebrates, whereas placental lactogen is produced by placenta in mammals. PRL has more actions than all other pituitary hormones combined. The initial step in the action of PRL is the binding to a specific membrane receptor, the PRL receptor (PRLR). Similar to the ligand, the PRLR has also been shown to be a member of the same family as the GH receptor, and as well part of the larger class of receptors, known as the class-1 cytokine receptor superfamily.

In this review, we will discuss many of the molecular steps by which PRL exerts its various functions. This includes the sites of PRL synthesis, PRL structure, the interaction of PRL with its receptor and the main signaling cascades activated in target cells. In an recent review we have expanded the original list of 85 different actions of PRL in vertebrates to more than 300 separate biological functions. In addition, we have recently generated a mouse model deficient in the PRLR which allowed us to detect unexpected phenotypes, indicating that PRL is involved in other functions than those reported to date. The main phenotypes of PRLR knockout (KO) mice will be discussed, along with those described for other related knockout.

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Casein gene locus control region?

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The organization of the bovine, murine and human casein gene loci was shown to be highly conserved, with respect to order, orientation and distance of the genes. Expression analysis of the individual bovine casein genes in transgenic mice have shown that regulatory elements capable of driving high level, tissue- and stage-specific expression, at levels similar to or even exceeding those observed in bovine, are present in the as1- and the b-casein transgenes used.

These studies also showed that sequences required for efficient expression of the bovine as2- and k-casein genes such as enhancers or a Locus Control Region (LCR) are not closely linked to these genes and are possibly situated elsewhere in the casein locus.

It is conceivable that the expression of the genes in the casein gene locus is controlled by common cis-acting regulatory elements. This elements are possibly situated in the a/b region based on the following facts:

1) the casein genes are clustered,
2) they are coordinately expressed in a tissue- and developmental-stage specific fashion,
3) the calcium sensitive casein genes are evolutionary related and share the same set of *cis*-acting regulatory elements in their proximal 5' flanking regions. The k-casein gene is not related to these genes but has a functional relation as the expression of its gene product is essential for casein micelle formation and stability,
4) the absence of expression of the individual bovine as2- and k-casein genes in transgenic mice suggests that these casein genes lack the regulatory elements in their proximal flanking sequences required for proper regulation of expression,
5) the overall organization of the casein gene locus (especially the a/b region) is conserved between species.

Preliminary analysis of DNase I hypersensitive regions (HS) in lactating mammary gland tissue of mouse and cow has revealed the presence of HS in the a/b intergenic region. Comparative sequence analysis between mouse and cattle identified conserved sequences in the a/b intergenic region. 71% of the bovine and 52% of the mouse intergenic region consists of repetitive sequence accounting for most of the difference in size between the mouse and bovine intergenic region, 10 and 19.6 kb, respectively. Studies to identify *trans*-acting factor binding sites, possibly involved in casein locus regulation are underway.

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The optimal use of IRES (International ribosome entry site) in expression vectors

Joe Attal, Marie-Claire Théron, Louis Marie Houdebine

In higher eucaryotes, natural bicistronic mRNA have been very rarely found so far. The second cistron of bicistronic mRNAs is generally considered as untranslated unless special sequences named IRES (Internal Ribosome Entry Site) are added between the two cistrons. These sequences are believed to recruit ribosomes independently of a cap structure. In the present report, a new IRES found in the HTLV-1 genome is described.

A systematic study revealed that the IRES, but also the poliovirus and the encephalomyocarditis virus (EMCV) IRES works optimally when they are added about 100 nucleotides after the termination codon of the first cistron. Unexpectedly, these IRES became totally inefficient when added after 300-500 nucleotides spacers. This result and others are not compatible with the admitted mechanism of action of IRES. The IRES appear to be rather potent translation stimulators. Their affects are particularly emphasised in cells in which the normal mechanism of translation initiation is inhibited. For these reasons, we suggest to call IRES Rescue Translation Stimulators (RTS).

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Transgenic pigs as bioreactors: a comparison of carboxylation of glutamic acid in recombinant human protein C and factor IX by the mammary gland

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The mammary gland of transgenic livestock can be used as a bioreactor for producing complex therapeutic proteins. However, the capacity for making a given post-translational modification upon any given polypeptide is uncertain. For example, the efficiency of gamma-carboxylation of glutamic acid in the amino terminal regions of recombinant human protein C (rhPC) and recombinant human Factor IX (rhFIX) is different at similar expression levels.

At an expression level of about 200 µg/mL in the milk of transgenic pigs, rhFIX is highly gamma-carboxylated as indicated by pro-coagulant activity and amino acid sequencing. However, only about 20-35% of rhPC has a native, gamma-carboxyglutamic acid-dependent conformation and anti-coagulant activity. Thus, this work provides an example of apparent differences in substrate specificity between two homologous proteins to the endogenous carboxylase of porcine mammary epithelium which leads to varying degrees of post-translational modification.

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Fusion protein approach for expressing biologically highly active proteins in milk of transgenic animals

Juhani Jänne, Veli-Pekka Korhonen, Juha-Matti Hyttinen, Leena Alhonen, Minna Hendolin

We have expressed human granulocyte-macrophage colony stimulating factor (GM-CSF) and human erythropoietin (EPO) genomic sequences under the control of bovine alpha-S1-casein promoter in milk of transgenic mice. Even though both fusion genes contained same bovine regulatory sequences, the GM-CSF-derived sequences were expressed in the range of mg per ml while those derived from EPO yielded milk concentrations that were 3 to 6 orders of magnitude lower than the levels of GM-CSF. Simultaneous integration of GM-CSF and EPO sequences resulted in a partial rescue of EPO expression. Transgenic mice efficiently expressing human GM-CSF showed only minimal unwanted effects, such as small increase in white blood cells, whereas EPO expressing mice suffered from severe polycytemia even at EPO levels of ng per ml. The increase in red blood cells was not only limited to lactating females but also virgin females and transgenic males showed distinct signs of enhanced erythropoesis. To avoid EPO-induced side-effects, we constructed a fusion gene in which human EPO cDNA was inserted into 5th exon of bovine beta-lactoglobulin gene with a linker sequence encoding the cleavage site for bacterial IgA protease. This fusion gene was efficiently (up to 0.5 mg per mL) expressed in milk of transgenic mice and rabbits. The beta-lactoglobulin/human EPO fusion protein isolated from milk retained less than 15% of the EPO activity that was, however, fully recovered upon treatment the milk with IgA protease. Transgenic males or virgin females did not have any signs of stimulated erythropoesis, yet lactating females showed a transient increase in their hematocrit values. Even though not fully applicable to human EPO, these results indicate that structurally highly conserved growth factors can be expressed at high levels in a form of biologically inactive or less active fusion proteins.

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Transgenic rabbits for the production of biologically active recombinant proteins in the milk

Fidel Ovidio Castro, José Limonta, Alina Rodríguez, Alina Aguirre, José de la Fuente, Anselmo Aguilar, Boris Ramos, Orlando Hayes

The use of live bioreactors for the expression of human genes in the mammary gland of transgenic animals is one of the most cost-effective ways for the production of valuable recombinant therapeutic proteins. Among the transgenic species used so far, rabbits are good candidates for the expression of tens to hundreds of grams of complex proteins in the milk during lactation. The lactating mammary gland of rabbits has proven to be effective in the processing of complex proteins. In this work, the potential use of rabbits as bioreactors is discussed based on our results and the published data.

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Dolly, Polly and Other "ollys": likely impact of cloning technology on biomedical uses of livestock

Alan Colman MA

The idea of generating transgenic livestock which secrete into their milk large quantities of proteins for therapeutic use, was pioneered in the late 1980's with the disclosure of the production of a number of transgenic sheep.

One particular animal, a sheep called Tracy, produced milk where over 50% of the protein consisted of human alpha 1 anti-trypsin. Sheep-derived pro-

tein has now entered clinical trials for cystic fibrosis (United Kingdom, USA) and congenital emphysema (United Kingdom). There are many other examples where this technology is making inroads into more traditional ways of making biopharmaceuticals. However, although robust, this technology has several limitations, including an inability to allow targeted insertion/modification of the animal genome, long timelines to production flocks/herds, and the rather unpredictable expression levels seen when different transgenic founders are compared. We believe that there is now a technical solution to all of these problems.

Dolly is a high profile example of a new technology comprising the generation of identical animals from cultured somatic cells. This work has many implications. In the commercial context, the real benefits of this advance will be seen when genetically engineered somatic cells are shown to be suitable nuclear donors, and particularly when the manipulations are targeted to pre-determined sites in the host cell genome. The first objective has now been achieved with the birth of Polly, a cloned sheep which contains the human gene encoding Factor IX, a protein involved in preventing haemophilia.

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In vitro development of rabbit nuclear transfer oocytes reconstructed with fetal fibroblast cells

Boris Ramos, Anselmo Aguilar, Orlando Hayes, Fidel Ovidio Castro

With the birth of Dolly, the lamb, new avenues of research were open for biologists, specially for those involved in gene transfer programs in farm animals. Since them, a lot of efforts have been pursued to obtain somatic cloned animals in laboratory and livestock species. The successes obtained in ruminants and mice have further encouraged for the widest investigation in this field.

In our group we are involved in a gene transfer program in rabbits for the production of recombinant proteins in their milk, although this is feasible relatively easy by conventional methods of gene pronuclear microinjection, it is not possible to target the integration event in the injected embryos. For this reason we studied the possibility of making somatic nuclear transfer in rabbit oocytes, using foetal fibroblast cells as nuclei donors.

Foetal cells were isolated from 18-day old chinchilla fetuses, after sterile collection and enzymatic dissaggregation. Cells were cultured *in vitro* and frozen after the fourth passage. One week before the nuclear transfer, $4x10^5$ cells per 35 cm dish were plated and kept for additional 5 days in DMEM:F12 medium supplemented with 10% FCS, insulin and EGF. Two days before nuclear transfer cells were changed to the same medium but without serum for starvation. Cells were tripsinized, resuspended in DMEM:F12, plus 30% FCS and used in the experiments.

Oocytes were collected 14 h after hCG injection from New Zealand White donor rabbits. After hyaluronidase treatment and denudation, the oocytes were incubated in RD medium (RPMI:DMEM 1:1) plus cytochalasin, and enucleated under mild UV epifluorescent illumination. Immediately after that, one foetal cell was introduced and fused in manitol medium with the enucleated oocyte. Optimal fusion parameters included DC field strength of 2.2 $kVcm^{-1}$, 30 µsec, twice. Thirty minutes later oocytes were chemically activated with ionomycin and 6 DAMP.

Cleavage and blastocyst development was scored at day 1 or 6 of culture respectively. The following figures were obtained. Survival to enucleation: 90%, fusion rate: 45%; cleavage: 82%. No blastocyts were obtained in the experiments. In the partenogenetic control group of non-enucleated, activated oocytes, the blastocyts rate was 55%, while no development was achieved in enucleated and activated reconstituted control oocytes. Our results showed that reconstituted oocytes after fusion with foetal fibroblast cells and chemical activation were capable of initiating early cleavage but failed to develop further into blastocyst. The reason for this is not clear at present and constitutes the subject of deeper investigation.

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Isolation and partial characterization of bovine fetal cells for nuclear transfer experiments

Orlando Hayes, Boris Ramos, Fidel Ovidio Castro

With the aim to develop cell cultures as potential nuclei donors for cloning experiments, we isolated and partially characterized fetal primary cell cultures from cattle. A 55-days old male fetus was surgically collected by histerectomy from pregnant cattle of the breed Crimosin (Creole x Limousin) F1. After washing in ethanol and PBS supplemented with antibiotics, the fetus was decapitated, eviscerated and the limbs and tail discarded. Tissue explants were made from skin and muscle. The remaining tissues were digested with collagenase in a Hepes-buffered medium.

Explants were cultured for a period of 8 days for muscle and 1 month for skin in DMEM:F12 medium supplemented with 10% FCS, insulin and EGF. After this, clear outgrowths were visible under the microscopy. The tissue was discarded and the outgrowths subcultured and frozen for further studies. The collagenase-diggested tissue was passed and frozen too. In all cases cells were frozen after the first passage. Immunohistochemical studies in situ with monoclonal antibodies anti-keratins 10,17,18 and 19 and anti-actin gamma and alpha (Zymed, San Francisco, USA), further verified the origin of the established primary cultures. The doubling time was scored in cell cultures after at least five subsequent passages.

All three primary cultures i.e muscular, skin and mixed tissue were transfected with a reporter gene encoding bacterial b-galactosidase under the con-

trol of CMV gene promoter, and revealed histochemically with an X-gal assay. For transfection, we used the polycation, polyethilenimine as vehicle. The result of the transfection showed that high efficient transfection was obtained in all three primary cultures. However, there was a background of ß-galactosidase activity in all samples, being stronger in the culture of mixed tissues. Nevertheless, it was possible to distinguish the endogenous mild background, from the cells expressing b-galactosidase. The later turned deep blue after staining. For further studies we recommend the use of another reporter gene in order to avoid misinterpretation of the results due to background activity of the unspecific hydrolisis of galactose. In summary, we established primary cultures of muscle and skin of a calf fetus, studied the behavior of these cells *in vitro* and showed their ability to incorporate foreign DNA after transfection. The next step will be the karyotyping of the cells and their use in nuclear transfer experiments for cloning in cattle.

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The use of yeast artificial chromosomes in transgenic animals:

Expression studies of the tyrosinase gene in transgenic mice

Patricia Giraldo, Estela Giménez, Lluis Montoliu

A yeast artificial chromosome (YAC) harboring the mouse tyrosinase gene completely rescued the albino phenotype in transgenic animals, as reported before. The YAC (250 kb) encompassing the tyrosinase gene was expressed faithfully in transgenic mice, in a position-independent and copy-number dependent manner. New transgenic mice were generated later with YACs carrying several deletions in the mouse tyrosinase locus to define the sequences important for this correct qualitative and quantitative expression pattern. New transgenic mice were generated later with YACs carrying several deletions in the mouse tyrosinase locus to address the *in vivo* relevance of a cell-specific DNAse I hypersensitive site (HS) located -12 kb upstream of this gene. Wild-type level of expression was observed only when the YACs transferred contained this HS. Constructs in which the HS was deleted gave rise much weaker expression and variable patterns of expression. Moreover, variegation and inherited somatic mosaicism could be observed in transgenic mice carrying YACs in which the HS had been deleted or replaced. This mosaicism trait was present in the two cell types where this gene is thought to be specifically expressed: melanocytes found in the skin, iris and other parts of the body and, retinal pigment epithelium cells, thus resulting in patches of pigmentation in skin and retina. This HS region appears to harbor essential regulatory elements for the correct expression of the tyrosinase gene. Furthermore, it behaves as a locus control region (LCR) protecting it from position effects and mosaic expression occurring in its absence. We conclude that this LCR suppresses the variegated expression of the YAC transgene and has a crucial role in the establishment and maintenance of the expression domain of the mouse tyrosinase gene. We will discuss these data as a general explanation, which could apply for the optimal expression levels routinely, reported with YAC-based transgenic animals.

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