Risks and benefits related to livestock cloning applications

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Foreword

Animal cloning can present the advantage of participating in genetic progress by promoting the dissemination of validated genomes within herds. Scientists, breeders and genetic experts worldwide, particularly in the United States, Japan and China, have been conducting numerous research programmes with the ultimate aim of being able to use cloning techniques for livestock breeding and animal production. In Europe, the bioethical debate associated with the use of these techniques has led to research programmes being scaled down significantly. Several countries, including France, nonetheless remain present in the field of animal cloning, which, despite its imperfections, continues to interest cattle breeders in particular.

The consumption of products derived from cloned animals or their progeny now appears to be technically possible. An assessment of the potential risks to consumers and herds is therefore necessary.

Until now, the consumption of products derived from cloned animals has been the subject of a de facto worldwide moratorium. Preliminary but convergent studies indicate that none of the measurable overall parameters (composition of meat and milk, presence of toxic or allergenic substances, behaviour, health, reproduction, etc.) suggest that a cloned animal is abnormal. However, these conclusions are based on only a very restricted number of cases, mainly in farm animals.

In the United States, the Food and Drug Administration carried out an assessment of the nutritional risks of products derived from cloned animals and estimated that the meat and milk from these animals was just as safe as that from conventional animals. However, a US scientific committee requested that publication of this report be deferred, deeming that the evidence provided was insufficient. The health authorities in Australia and New Zealand compiled a review of available data relative to the safety of products derived from cloned animals and requested that a cautious approach be adopted before concluding that products derived from conventional animals and cloned animals are equivalent.

Any technological advance involves both risks and benefits and animal cloning is no exception to this rule. Afssa (the French Food Safety Agency) wanted to review the state of knowledge in the field of animal cloning and to assess the risks relative to the consumption of products derived from cloned animals. In addition to these food safety aspects, genetic aspects, along with those related to genetic diversity, animal health and welfare were also considered to be important factors to be taken into account in this assessment.

Contents

Introduction

Both biologists and livestock breeders seek animals presenting a broad genetic diversity. This provides biologists with models that can be used to study biological functions and certain human diseases. And breeders benefit from animal lines that are the most suitable for human requirements, using genetic variability to select their breeding animals.

To achieve these aims, two approaches are traditionally used: sexual reproduction and the selection of the best individuals. Considerable progress has been made in the last hundred years to optimise these approaches to improve animal lines. Controlling reproduction in animals is based on the following methods: choice of sires, artificial insemination, embryo collection and transfer, oocyte collection and maturation followed by in vitro fertilisation and embryo transfer, long-term storage of gametes and embryos. As for selection, this is becoming ever more precise as choosing animals from a population, particularly breeding animals, is increasingly no longer based on overall observation of individuals but on specific biochemical measurements and the chemical structure of the chromosome regions carrying the genes of interest [Quantitative Trait Loci (QTL) or marker genes].

Sexual reproduction, by its very nature, generates genetic diversity since it involves random redistribution of parental genes. This leads to the birth of individuals which, within a given species, all have the same genes but in different versions. These combinations mean that every individual is unique. Sexual reproduction is therefore a lottery, which promotes maintenance of the species. The latter thus possesses a diversity of individuals, some of which are well equipped to survive environmental changes and others much less so.

The effect of selection is a reduction in the random component of sexual reproduction. A method of reproduction capable of bypassing sexual reproduction is therefore, theoretically, a way of reducing the element of chance. Cloning is a way of meeting this objective.

Although asexual reproduction is the rule in microorganisms, it is also common in plants, naturally through layering and artificially through propagation and cell culturing. In the latter case, the cells of plant organs are transformed into embryo cells through straightforward culturing. These methods are widely used in research and plant production. In animals, cloning is currently only possible through the mechanical transfer of the nucleus of a cell, which is differentiated to a greater or lesser extent, into the cytoplasm of a previously enucleated oocyte.

Animal cloning is a complex and still highly empirical technique. Theoretically, it can be employed for the purposes of conducting basic research, to accelerate genetic progress, to obtain cells capable of regenerating damaged organs or to produce individuals genetically identical to the nucleus donor.

Despite its limited efficacy, the cloning technique could already be used to perpetuate high-value breeding animals within herds. It is therefore possible that, in the relatively near future, products (milk, meat) derived from the progeny of animals born as a result of cloning (and not the clones themselves) could be offered to consumers. It is also important to note that the genetic improvement of livestock via gene transfer, still at an experimental stage, is increasingly frequently based on the cloning technique.

This new technique still involves a number of uncertainties. However, in principle, there is no reason for products derived from clones and their progeny to be any more risky to consumers than conventional products, since they come from animals for which the zootechnical performance is known and since cloning, by its very nature, reduces the hazards of conventional reproduction. However, a relatively high proportion of clones present various metabolic disorders at birth, which usually disappear in the following weeks.

This report presents a review of cloning techniques and an assessment of the risks that products derived from cloned animals could carry in terms of potential toxic and allergenic effects. This report also examines the consequences of the use of these techniques on the health and welfare of animals, in the short and long term, and also the consequences for the herd of a reduction in genetic diversity resulting from reproduction by cloning. Finally, some of the socio-economic problems are outlined, to put the overall question into context. None of the ethical issues are dealt with.

Several reports on the risks that could result from the use of reproductive techniques, including cloning, have been published in the last 5 years (AFSSA 1999; NAS 2002; Pew initiative 2002; ICSU, 2003; Seamark 2003).

It is also interesting to note that a symposium held at Jouy-en-Josas in November 2003, at the initiative of INRA (French National Institute for Agronomic Research) and the OECD, reviewed the various studies aimed at assessing the risks that could result from the consumption of various products derived from cloned animals. The proceedings of this symposium are published in the June 2004 edition of the *Cloning and Stem Cells* journal.

1 Cloning

1.1 THE PRINCIPLE OF CLONING

The development of a sexual living organism involves a series of steps, the first of which is fertilisation. The resultant zygote formed contains a single cell, which contains two chromosomal copies, one present in the oocyte and the other supplied by the spermatozoon. This is called a diploid cell since it contains two chromosomal copies, like the cells of all the organs resulting from it, termed somatic cells.

The first cell forming the embryo divides to form two then four cells, etc. Up until the 4-cell stage, each cell is totipotent. This means that each of these cells has every potential and, in particular, is capable of ensuring the complete development of the organism when placed in suitable conditions, i.e. their presence in the uterus in the case of animals. Beyond this stage of development, the embryo cells lose their totipotentiality and become pluripotent. This means that none of these cells alone can ensure the complete development of an organism, but that each of them can participate, indifferently, in the formation of any organ, on the express condition that they are combined with other cells, as is the case in the embryo at the blastocyst stage. As they continue to divide, the cells gradually specialise. This specialisation is termed differentiation. They are then multipotent, which means that they are now only capable of participating in the formation of certain well-defined organs and tissues (for example, the cells of the bone marrow, which give rise to all the blood cells, red cells and white cells). For cells, the last stage consists in specialising completely in order to fulfil the functions allocated to them in each organ or tissue in which they are located.

This process, known as differentiation, is deemed to be irreversible, insofar as a differentiated cell, either multipotent or pluripotent, cannot spontaneously become totipotent once more (figure 1).

From pluripotent and multipotent cells, it is possible to establish cell lines, called embryonic stem cells in the first case and organ stem cells in the second. By definition, a cell is capable of dividing identically a very large number of times and of differentiating if necessary. In the course of embryo development, stem cells differentiate spontaneously under the influence of inducers coming into contact with them. The pluripotent cells of embryonic stem cell lines can differentiate when they are reintroduced into an early embryo or when suitable inducers are added to their culture medium. Embryonic stem cells are therefore a potential source of multipotent or differentiated cells to regenerate damaged organs in patients. Organ stem cells amplified in vitro can also contribute to the regeneration of organs. In certain situations, the stem cells of an organ can be converted into stem cells of another organ, via a process known as transdifferentiation.

Sex cells or gametes are formed from somatic cells derived from pluripotent cells by a short circuit involving only a small number of cell divisions. Sex cells have become haploid (only now containing one chromosomal copy) and diploidism is restored by fertilisation (figure1).

Sexual reproduction creates a new genome following the formation of gametes and fertilisation. In fact, the homologous chromosomes exchange their genes randomly during the formation of gametes and these new chromosome versions are also distributed randomly to form haploid gametes. The meeting of gametes during fertilisation adds another random component to sexual reproduction. These mechanisms lead to a genetic diversity enabling species to adapt to environmental pressures and selection. They are exploited by breeders who artificially encourage the production of animals meeting their requirements.

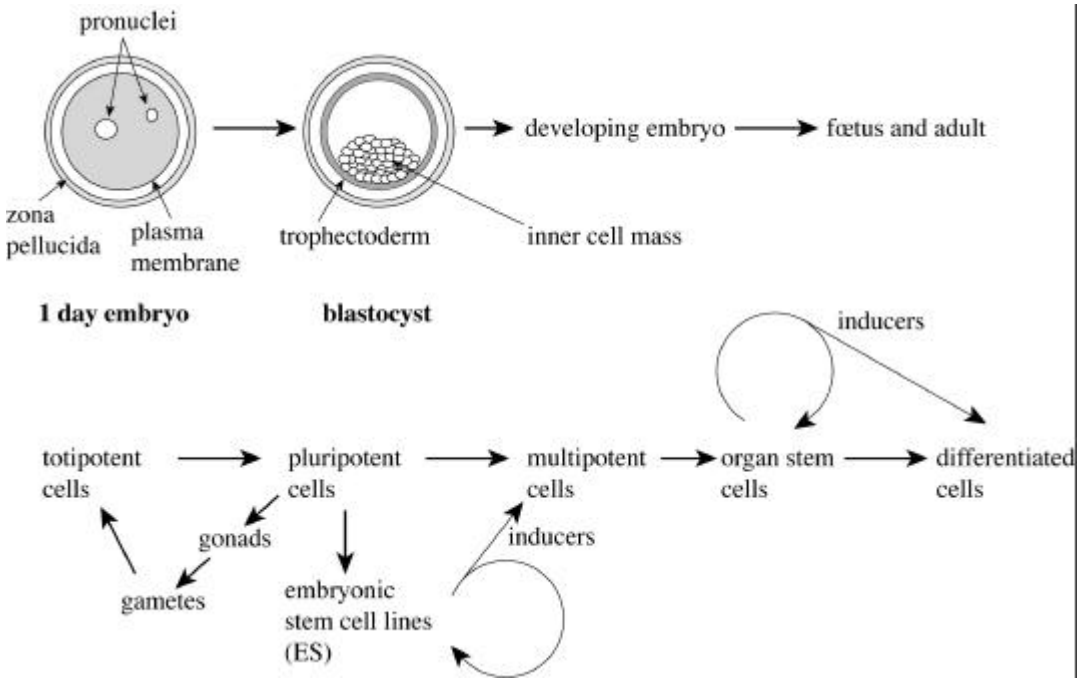

Figure 1: The various stages of development. The cells progressively and irreversibly lose their potentiality by differentiating. The gonads and sex cells are formed from pluripotent cells by bypassing the general differentiation process.

The reproduction of genetically identical individuals is generally the rule in microorganisms, and the same is true for mushrooms, which can reproduce from their mycelium; it is relatively common in plants in reproduction from bulbs or tubers or via multiplication by layering, or from rhizomes or stolons. Propagation and grafting also avoid the process of sexual reproduction and enable the mass multiplication of plants with known phenotypic properties. These “plant multiplication” practices are made possible by the existence in plants of meristems, which maintain an undifferentiated state and ensure their growth. This type of reproduction does not occur in higher animals.

Embryo cloning is another way of avoiding the sexual process to obtain normal organisms that are genetically identical to their progenitors (figure 2).

Cloning in plants

Cell cloning in plants was performed for the first time around 50 years ago. This technique consists in redifferentiating differentiated cells of the plant in vitro. Relatively simple culture media enable these cells to become totipotent again and hence, theoretically, to become capable of each creating a plant genetically identical to the original one. In some cases, the use of this technique produces plants presenting various genetic abnormalities, limiting its wide-scale use. However, this method is used in a variety of species, including coffee plants and oil palms. In vitro propagation (or micro-propagation), often combined with a thermal treatment, is used to rid vegetative reproduction plants of the viruses spread by them. Since 1983, in vitro regeneration properties using single cells have enabled the production of transgenic plants, in which all the cells carry exactly the same transgene insertion (Robert *et a*l., 1994).

Towards animal cloning

The approach developed in plants was rapidly shown to be impossible to transpose to animals. It was therefore necessary to use more sophisticated methods to enable somatic cells to become totipotent once more. The method, which was defined around 50 years ago, consists in transferring the nucleus of a cell into the cytoplasm of an enucleated oocyte (figure 2).

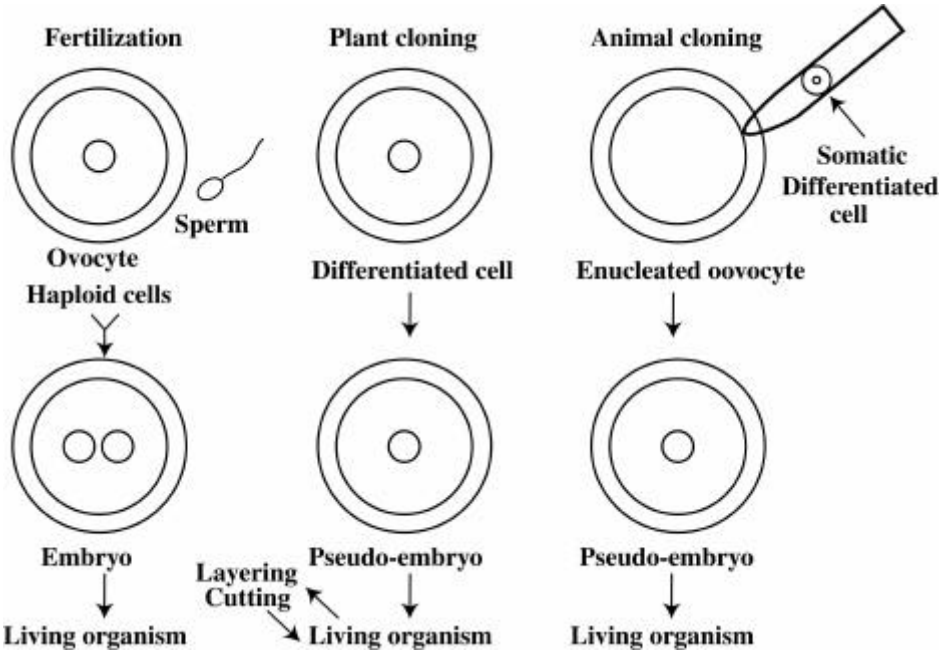

Figure 2: The various methods to obtain totipotent cells. Fertilisation leads to an embryo that develops according to the diagram in figure 1. ***In plants***, differentiated cells in the form of organs carrying meristems can produce normal organisms by layering or propagation. Differentiated plant cells can become totipotent again in vitro then differentiate to produce complete cloned individuals. ***In animals***, a return to totipotentiality is only possible through the action of the cytoplasm of an enucleated oocyte. In all three cases, the cell obtained is diploid and totipotent and hence capable of producing a living organism of normal appearance. The totipotent cells obtained by cloning must therefore be considered to be embryos in their own right.

The first successful nucleus transplantations were performed in amphibians by Briggs and King (1952), who obtained normal tadpoles following transplantation of nuclei from blastula cells into enucleated frog's eggs. This technique was used mainly in amphibians to study modifications in the nucleus of somatic cells during cell differentiation during development in Xenopus toads and frogs (Gurdon, 1986; DiBenardino, 1987). In fish, the first nucleus transplantation trials were performed by Chinese scientists in the 1970s, but did not lead to any conclusive results. As early as 1979, Gasaryan et al. performed embryonic nucleus transplantation into the oocytes of loach and obtained nuclear transplants which developed as far as the hatching stage, but not beyond. In parallel, several Chinese teams (Yan, 1989, 1998) produced nucleo-cytoplasmic hybrids by transplantation of nuclei from one species into the enucleated oocytes of another species. This pioneering research conducted in loach and Cyprinida was not subsequently developed in other species.

These experiments were extended to sheep in 1986 with the aim of accelerating genetic progress in ruminants. This success is still too limited to give rise to zootechnical applications. The yield of the method was low and only the uncultivated pluripotent cells of early embryos (morula-blastocyst), the genetic heritage of which was not individually known, led to the birth of live animals. There was a major breakthrough in 1996, when cloned lambs were produced from embryonic pluripotent cells kept in culture for several weeks (Campbell *et a*l., 1996). Shortly afterwards, the same experimental conditions led to the birth of lambs cloned from differentiated foetal and adult cells (Wilmut *et a*l., 1997). It was therefore proved that the genome of differentiated cells could lead to the birth of viable animals.

More recently, these cloning techniques have also been developed in fish. Thus, Wakamatsu et al. (2001) obtained fertile nuclear transplants from the nuclei of the embryonic cells of medaka and demonstrated the Mendelian transmission of marker genes in the progeny of these transplanted cells. Nuclear transfer from fibroblasts obtained by long-term culture (13 passages) was performed in 2002 in another model species, the Zebrafish (Lee *et a*l. 2002).

1.2 CLONING APPLICATIONS

1.2.1 Research applications of cloning

Despite the limited successes of current cloning techniques in animals, several applications are possible or are in the process of being evaluated. Cloning by nuclear transfer offers unprecedented possibilities for the study of genetic programming and cell differentiation mechanisms. The creation of cloned laboratory animals makes it possible to more accurately assess the properties of new substances of therapeutic interest. Cloning is a technique that has been adopted by scientists to add foreign genes in ruminants and to replace genes by homologous recombination in species other than mice. Indeed, in practice this is the only species that can be used to obtain germinal chimeras, transmitting the mutations induced in pluripotent ES or EG cells to their progeny (figure 3).

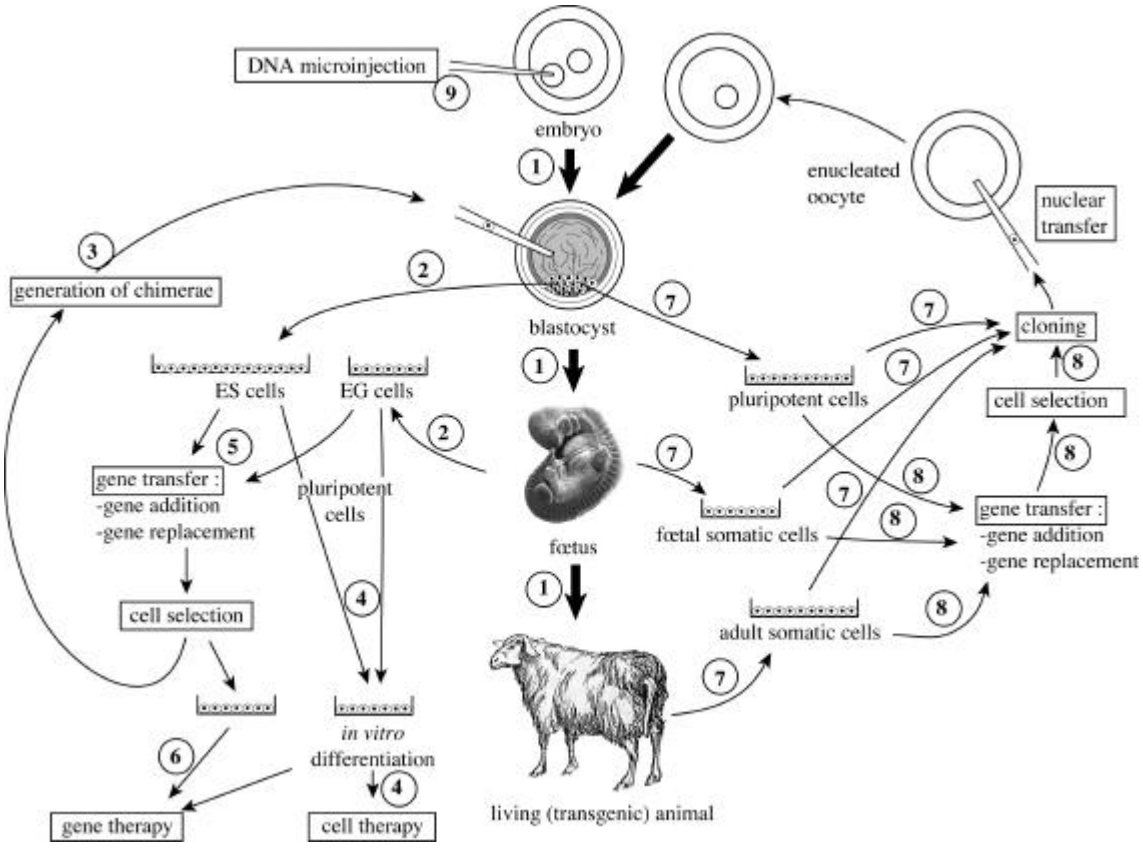

Figure 3: The possible relationships between cloning, transgenesis, cell therapy and gene therapy. 1) Normal embryo development; 2) Establishment of pluripotent cell lines from the internal cell mass of blastocysts (ES cells) or foetal gonads (EG cells); 3) These pluripotent cells, reintroduced into a recipient blastocyst, participate in the development of all organs and produce chimeric animals; 4) The pluripotent cells can differentiate in vitro and be introduced into patients to regenerate damaged organs (cell therapy), (cell therapy can also be performed using organ stem cells or already differentiated cells); 5) Genes can be transferred into pluripotent cells, subsequently used to produce transgenic chimeric animals (this method is mainly used in mice for gene replacement); 6) Pluripotent cells having received a foreign gene can differentiate in vitro and be used for gene therapies (gene therapies are generally performed using differentiated somatic cells); 7) Pluripotent or differentiated cells from foetuses or adults can be used for cloning by nuclear transfer; 8) Cells which have received genes can be used to create transgenic cloned animals; 9) Genes can be introduced into an embryo by micro-injection to produce transgenic animals.

A recent study showed that, by successive homologous recombinations followed by cloning in the same cow, it was possible to inactivate the two alleles of two genes, one encoding the PrP protein, which plays a major role in the development of bovine spongiform encephalopathy (Kuroiwa *et al*., 2004).

1.2.2 Zootechnical applications of cloning

By making it possible to obtain genetically identical animals, cloning could offer the possibility of "bringing back to life" pets. Cloned cats have been created for this purpose. Other animals, such as dogs, will probably be produced in the same way in the not too distant future. The reproduction of these types of animals by cloning represents a potentially valuable market in financial terms.

Cloning of show jumping horses is currently under way. This is of particular interest insofar as the best of these animals are generally castrated males. Castration before puberty makes these animals docile, but also sterile. Their reproduction is (therefore) only possible by cloning.

In addition, cloning could theoretically help to save animals threatened with extinction. The nuclei of cells harvested from a few individuals of the species could be transferred into the cytoplasms of enucleated oocytes from a related species. In this way, a cloned mouflon has been born following nuclear transfer to enucleated sheep oocytes (Loi *et al.,* 2001). This success is probably due to the fact that the sheep is derived from the mouflon species.

Finally, cloning makes it possible to envisage the dissemination of genetic progress, through the cloning of breeding animals with phenotypic traits of interest to breeders. Cattle are currently the only species for which reproduction by cloning is being considered.

It is this last application, which implies the consumption of products derived from cloned animals or their progeny, that this report focuses on.

1.3 CLONING TECHNIQUES

1.3.1 Introduction

The nucleus of the spermatozoon, the DNA of which is coated with protamines and which is temporarily no longer capable of replicating or expressing the genes that it contains, rapidly undergoes profound changes in the hours following fertilisation. In contact with the oocyte cytoplasm, the nucleus of the spermatozoon loses its protamines, which are replaced by histones and regulatory nuclear proteins. The core decondenses to become visually similar to that of the oocyte less than 24 hours after fertilisation. The DNA of the oocyte and spermatozoon nuclei can then simultaneously replicate to produce the first cell division of the embryo. In the very first days of development (from 1 to 4 days depending on the species) the genome begins to be transcribed. The cytoplasm of the oocyte has therefore been capable of programming the genome of the spermatozoon, making the immediate expression of a large number of genes possible, and also the future expression of all the body's genes during foetal development and in adulthood. During gametogenesis and in the period following fertilisation until the blastocyst stage, the DNA of the embryo undergoes massive demethylation, enabling the expression of a large number of genes. From the time of "hatching" and during implantation, the DNA remethylates, but in a selective manner. This mechanism participates in the selection of the regions of the genome which will retain their capacities to transcribe the genes they contain. For some genes, the region where they are located is methylated on one of the parental chromosomes and not on the other. Non-methylated genes are the only ones that remain activable. This phenomenon, which is more or less transmissible from one generation to another, is known as genetic imprinting. The extinction of a gene does not therefore depend on DNA mutations in these situations but on its local inactivation. For this reason, this type of phenomenon is termed epigenetic.

The experimental fusion of cells leads to hybrid cells being obtained, which, to a greater or lesser extent depending on the case, retain the expression spectra of the genes of the two cells. Hybrids between somatic cells and pluripotent ES cells mainly express the genes of the ES cells, whereas the specific genes of the somatic cells are extinguished in these hybrid cells. The oct4 gene, expression of which is restricted to pluripotent cells, is thus reactivated in hybrid cells in which one of the partners is an ES cell. The same is true for the genes of the X chromosome, silenced during development. The methylated genes responsible for the phenomenon of genetic imprinting are also demethylated and reactivated. It is therefore considered that oocytes and pluripotent cells have a dominant character as far as genetic programming is concerned (Jouneau and Renard, 2003). It is this property that has been exploited for 50 years to obtain animal clones.

1.3.2 Oocyte enucleation

To be able to benefit from the genetic programming properties of the cytoplasm of the oocyte, the nucleus is first of all mechanically removed by aspiration. The polar body containing the set of chromosomes ejected by the cell to form the haploid gamete and which is located between the pellucid zone and the membrane is aspirated at the same time.

This operation is traumatic, not only due to the relative mechanical violence that it involves, but also because it is accompanied by the removal of around a third of the cytoplasm. This reduces the oocyte's programming capacities by the same proportion. Compensatory additions of oocyte cytoplasm somewhat reduce the extent of this phenomenon. The addition of exogenous oocyte cytoplasm no longer appears to be necessary when the nucleus of the cytoplasm is not removed by aspiration but by splitting the cell using a cutting blade to separate the nucleus from the rest of the oocyte.

1.3.3 Choice of nucleus donor cells

The origin of nucleus donor cells has a very significant impact on the efficiency of cloning. The pluripotent cells of uncultivated embryos give the best cloning yields. This yield falls significantly when these cells have been cultivated for several weeks. The efficiency of cloning decreases again when the donor cells are taken from a foetus and, above all, when they are taken from an adult (Wilmut *et al*., 2002; Hiiragi and Solter, 2005).

It therefore appears that the more differentiated the nucleus donor cell is, the more difficult it is to effect a return to totipotentiality. It is important to note that the use of B and T lymphocyte nuclei has enabled the cloning of mice. The yield of the operation was particularly low, but these results have removed an ambiguity. In fact, it is always possible that the low success rate of cloning using primary cells is due to the presence of organ stem cells which are incompletely differentiated and therefore more likely to regain a totipotent status. If it does exist, this phenomenon no longer appears to be possible using B and T cell clones, in which both the genes of immunoglobulins and those of receptors, which are only rearranged in this type of cells in differentiated state, can be unambiguously identified (Hochedlinger *et al*., 2002).

The cell type of the nucleus donor cell is also important. Cumulus cells in adults and skin fibroblasts in foetuses are some of the best nucleus donors without it having been possible to determine the reasons for this. Current data indicate that the type of cells used as nucleus donors has an influence on cloning efficiency but not on the physiological traits of the animals born following nuclear transfer.

The physiological situation of nucleus donor and recipient cells is also very important. This is particularly true with respect to the phase of the cell division cycle of the two cells. Several strategies are possible, without it having been possible to demonstrate whether one of these is incontestably the best (Wilmut *et al.,* 2002; Li *et al.,* 2003).

It is important to note, furthermore, that some oocyte or nucleus donor animals systematically lead to better cloning yields than others. The mechanisms determining these properties, and which appear to be genetic in origin, are unknown (Powell *et a*l., 2004).

The health of the cloned animal will closely depend on the health status of the donor animal from which the nucleus of a cell will be used to obtain the clone. Similarly, it will depend on the health of the recipient female. Any pathological event during gestation could have an impact on the health of the foetus and of the cloned young animal. In particular, the presence in the nuclei of cells used for cloning of the genomes of certain viruses in the latency phase (e.g. the Herpes virus) or incorporated in the cell's genes (e.g. retrovirus) can have a direct impact on cell development, the foetus or the young clone after birth. All these elements must therefore be taken into account when selecting donor cells and hence the donor or recipient animal.

1.3.4 Nuclear transfer

Transfer of an isolated cell nucleus into the cytoplasm of an enucleated oocyte is mechanically possible but, in practice, it is only followed by embryo development in mice. It appears that the

architecture of the cytoplasm region, which surrounds the nucleus, plays an essential role and that manipulation of the isolated nucleus damages its integrity. However, it is this technique that is used in zebrafish and medaka without apparently posing any particular problem (the percentages for successful nuclear transfers are identical to those observed in higher vertebrates).

The nucleus donor cell is therefore introduced mechanically, by micromanipulation, between the pellucid zone and the membrane of the oocyte. This operation is followed by repeated treatment with an electrical field aimed at inducing fusion of the plasmic membranes of the enucleated oocyte and the donor cell. The new construction formed is very similar to a zygote. Treatment with an electrical field also has the effect of activating the new embryo so that it starts to develop. This electrical field triggers formation of pores in the membrane of the embryo, allowing ambient calcium to penetrate the intracellular compartment. This trick partially mimics the induction of calcium flows normally triggered by signals emitted by the spermatozoon during fertilisation.

Activation of the embryo derived from a nuclear transfer can also be triggered by the action of calcium ionophores (ionomycin or A23187), by the addition of DMAP (6-dimethylaminopurine) which is a kinase inhibitor capable of inducing inactivation of the MPF (M-phase promoting factor) of the oocyte (Hwang *et al*., 2004) or by the addition of protein synthesis inhibitors (cycloheximide) and a cytoskeleton inhibitor (cytochalasine).

The effects of these treatments are not always known. It has recently been indicated, for example, that DMAP, traditionally used by cell biologists to inhibit the phosphorylation of certain proteins, has very significant mutagenic properties.

The oocyte activation phase following nuclear transfer is a crucial stage in the success of cloning. The techniques used are not the same in all laboratories, without it always being very clear why scientists have chosen a particular method. In pigs, for example, one group performs double nuclear transfer. The first transfer is to an enucleated oocyte. The second consists in extracting the nucleus from the zygote and introducing it into a previously enucleated one-cell stage embryo. This protocol is based on the idea that reprogramming is conducted mainly in the presence of the oocyte's cytoplasm, whereas the authors believe that the cytoplasm of an embryo ought to be the most capable of guaranteeing the early development of the embryo.

For years, the rat has been considered to be a species particularly unsuitable for cloning. In this species, manipulation of the embryo is sufficient to induce its activation, which is then no longer controlled and is, as a result, asynchronous with the nuclear transfer itself. Artificial control of activation using a cdc2-specific kinase inhibitor, butyrolactone, has made it possible to clone rats (Zhou *et al*., 2003).

Rabbit cloning also owes its success to good control of the synchronism between activation of the oocyte obtained following nuclear transfer and its implantation in an adoptive female (Chesné *et al*., 2002).

1.3.5 Possible improvements in cloning techniques

It is widely accepted that clones are genetically identical to their genetic parents. The viability of clones and the various phenotypic and genotypic resemblances are not strictly sufficient to conclude that this is really the case. Indeed, nobody knows the genetic status of a somatic cell used as a nucleus donor. These cells are probably not strictly genetically identical. Studies based on comparison of multiple genetic markers are currently under way to compare the genomes of clones with those of their genetic parents (De Montéra *et al.,* 2004).

It is still reasonable to consider that the majority of clones derived from the same animal are genetically identical to each other and to the animal having donated its genome. However, since a number of genes are not correctly expressed, these clones are epigenetically modified.

In this field, it is still to be confirmed that the progeny of these clones are no longer epigenetically modified. Tests applied to clones to systematically measure the expression of a large number of genes have not yet revealed whether these same genes have regained a normal function in the progeny of clones.

Recent studies have shown that the artificial combination of two cloned embryos considerably increased the chances of development of these embryos and the survival of the neonates. This improved outcome of clones is in fact accompanied by a marked increase in early expression of the *oct4* gene, which is essential for embryo development (Houdebine, 2003). This study shows that the nuclear reprogramming process does not only occur during the first contact between the nucleus and the cytoplasm of the oocyte. It is therefore highly likely that reprogramming, which involves direct contacts between the embryo's cells, takes place over several days. These results could lead to improvements in cloning since eight times as many live mice have been obtained using this cloning method.

Some recent experiments show that the nuclei of fibroblasts can be reprogrammed using T lymphocyte extracts. Cells modified in this way express some of the specific functions of T cells (Hakelien *et al*., 2002). It is conceivable that cytoplasm extracts or isolated and well characterised factors may be able to dedifferentiate somatic cells to make them totipotent. The use of oocytes for cloning would then no longer be necessary. Without going so far, a recent experiment demonstrated that inoculation of permeabilised nucleus donor cells with extracts of mitotic-phase cells induces condensation of the chromosomes, which promotes the subsequent development of the clones (Sullivan *et al*., 2004).

In any case, oocytes represent a material limiting cloning in certain species, particularly in humans (Hwang *et a*l., 2004). Several years ago, the transfer of human nuclei to enucleated oocytes from cows led to blastocysts with a normal appearance. These experiments were repeated, this time using enucleated rabbit oocytes (Chen *et a*l., 2003). A significant proportion of embryos reached the blastocyst stage. Pluripotent cell lines were created using these blastocysts. Differentiated cell lines were then produced in vitro using embryo cells. These cells are similar to human cells. Although there are still uncertainties with respect to this technique and although it raises some specific ethical issues, it could offer a simplified method for the performance of therapeutic cloning in humans and cloning for various uses in animals (Wakayama, 2004). Various inter-species combinations between donor cells and recipient oocytes have been attempted and have led to the formation of blastocysts. One of the most recent attempts consisted in transferring chicken embryo nuclei into enucleated rabbit oocytes (Liu *et a*l., 2004). Another possibility may be the exploitation of a recent case demonstrating that ES cells (pluripotent) were capable of transforming into oocytes in vitro (Vogel, 2003). This process would make it possible to obtain a large number of genetically identical oocytes without the need for ovulation.

1.4 THE MECHANISMS OF CELL REPROGRAMMING

One of the specific characteristics of cloned animals is their limited capacity to survive. This observation raises a number of theoretical and practical questions, to which some partial answers have already been found. It appears to be increasingly probable that the various developmental disorders in cloned animals result mainly from incorrect reprogramming of the genome of the nucleus donor cell rather than genetic defects proper. Although it is still not possible to describe these phenomena in detail, it is possible to draw up an inventory of the biological disorders that are observed in clones. These affect the animals' lives to varying extents and must be related to the food safety qualities of the food products derived from clones.

1.4.1 The zootechnical characteristics of clones

The yields of cloning techniques are low in all species, but vary depending on the experimental conditions. The yield decreases according to whether the nucleus donor cells are embryonic (36%), foetal (15%) or somatic (5.5%). It appears that the more differentiated the cell from which it is derived, the less easily a genome can be reprogrammed.

One of the characteristics often observed with cloned embryos is that their development throughout gestation and at parturition is arrested. After cloning from somatic cells, 30% well conformed blastocysts can be obtained (versus 50% after in vitro fertilisation). Only 15% become foetuses and 5.5% adults.

It is widely accepted that these arrests in development are due to the non-availability of the genes required to go through the successive growth stages. This non-availability may be due to mutations inducing their inactivation by hypermethylation.

At parturition or in the following 6 months, around 30% of calves die. They present multiple morphological abnormalities. In particular, they are bigger than normal newborn calves, while their placenta is under-developed, with numerous morphological and functional abnormalities (Heyman *et al*., 2004; Chavatte-Palmer *et a*l., 2004; Wells *et a*l., 2004).

Some of the live offspring present no detectable abnormalities. However, others present a whole series of pathological signs, which are generally similar from one herd to another and from one laboratory to another. These abnormalities include: excess weight, hepatitis, lung diseases, obesity, low healing capacity, depressed immune system, impaired cardiovascular system, chronic hyperthermia, metabolic disorders, etc.

These syndromes often disappear in the first few months of life if the animals are given suitable treatment. The animals that reach adulthood are normal but their mortality is high and unpredictable. A proportion of these diseases are infectious, which may suggest that cloned animals have a weakened immune system. Clones do not appear to die prematurely following premature aging.

Clones can be divided into three categories: 1) those which present serious abnormalities and die during gestation and shortly after parturition; 2) those which present reproducible and reversible disorders, which could be the residual effects of unfavourable gestation conditions and, in particular, malfunction of the placenta; 3) those which do not present any observable abnormalities. For reasons that are unknown, abnormalities in newborn offspring obtained by cloning are more common in cows and sheep than in goats. They are rare in pigs.

The birth of clones is generally by Caesarean since parturition is not usually triggered spontaneously. It would appear that this is not due to an inability of the foetus to send signals to the placenta but more to the placenta itself, which is not sensitive to these signals.

Growth, reproduction and lactation of clones are normal. The same is true for the progeny of clones.

Various histological and biochemical tests are regularly conducted on clones. Measurement of certain growth factors and their associated proteins, such as IGFI, IGFII, IGFBP, hormones such as T4, GH, insulin, leptin, cortisol, ACTH, blood parameters such as corpuscular volume, haemoglobin, etc. do not reveal any differences with normal animals likely to indicate certain pathological conditions.

Certain clones present partial thymic aplasia and abnormally low antibody production.

Incomplete reprogramming of the genome of clones and weakening of the immune system of some of them may be liable to reactivate endogenous retroviral genomes. Indeed, genomes of this type are numerous in a lot of species. They are mostly inactivated by methylation of their DNA and then by successive mutations over time. Testing for the presence of bovine retroviruses has therefore been performed in clones. These tests have not revealed any propensity of clones to host active retroviruses.

It is interesting to note that certain well identified syndromes, such as obesity in the clones of a specific line of mice, are not observed in any of their progeny (Tamashiro *et al*., 2002).

Clones have been obtained from cloned mice and cattle. In the first case, the mice do not present any significant differences in comparison with control animals after six successive clonings (Wakayama *et a*l., 2000). Conversely, cloned cattle descended from clones present serious abnormalities and only survive with difficulty (Kubota *et a*l., 2004). Normal reproduction therefore appears to eliminate, to a large extent if not completely, the defects induced by cloning, whereas reproduction by cloning exacerbates them.

1.4.2 The genetic identity of clones

By definition a clone theoretically has the same genotype as its parent. The reality is less straightforward.

Scientists do not fully know the genome of nucleus donor cells. Data gathered progressively over the last five years or more indicate that many animals, including humans, are mosaic or chimeric. It is not unusual for two embryos to exchange cells or even to fuse. In this case, the offspring are chimeras. It appears that this phenomenon is more common than previously thought and that it could be amplified by reproductive techniques, often involving the transfer of several embryos to the same surrogate mother (Pearson, 2002). For their part, embryos transmit cells to their mother and then cells can become durably established in the mother's body (Barinaga, 2002).

A recent study demonstrated that a significant proportion of cattle clones presented abnormal chromosomal instability related to their poor state of health (Hanada *et al*., 2005).

An ongoing study indicates that cell exchanges between cloned embryos and their surrogate mother can be detected using mitochondrial DNA as a marker (Hiendleder *et a*l., 2004).

In mammals, the mitochondria only contain a small number of genes. These genes have a definite influence and several genetic diseases are known to result from a mutation in the mitochondrial genes.

During cloning, the nucleus donor cell passes on its mitochondria to the recipient enucleated oocyte and, consequently, to the clone. The mitochondria from the two sources are then present in the adult animals. This phenomenon does not occur during fertilisation. The mitochondria of the spermatozoon are not found in the embryo, which therefore inherits the mitochondrial genome from the mother only.

It is also important to note that the enucleated oocyte – nucleus donor cell pairing is not generally formed from the same partners. In fact, the same animal can donate a large number of nucleus donor cells but, due to their relative rarity, the oocytes must be taken from several females, not necessarily genetically related to each other.

There are therefore two separate reasons for clones not being genetically identical strictly speaking. It has been possible to use oocytes and donor cells from the same family. This protocol would only be able to meet the requirements of genetic selection to a marginal extent and has little chance of being regularly implemented.

Furthermore, somatic cells are not all genetically identical. Studies aimed at comparing the primary structure of the genome of clones and their genetic parents are under way (De Montera *et a*l., 2004). The primary structure of the genome of livestock is not known. Comparisons are therefore based on the observation of genome fragments using several different complementary techniques: AFLP (Amplification Fragment Length Polymorphism), MSAP (Methylation Sensitive Amplification Polymorphism) and RDA (Representational Difference Scanning).

The MSAP method is capable of demonstrating local differences in DNA methylation and hence epimutations potentially.

1.4.3 The epigenetic identity of clones

In vertebrates, DNA is demethylated during gametogenesis and early embryogenesis. It is selectively remethylated from the blastocyst stage. The DNA of somatic cells is largely methylated and this corresponds to the low number of active genes in each differentiated cell (Mc Lay *et a*l., 2003).

In order to be successful, cloning must demethylate DNA, as is the case in development following fertilisation. This is not generally the case (Pomerantz and Blau, 2004; Allegrucci *et al.,* 2004). The DNA of the X chromosome is intensely methylated in certain cloned cattle (Xue *et a*l., 2002). The degree of methylation of the DNA reflects the capacity of cattle and sheep clones to develop (Santos *et al*., 2003; Beaujean *et al*., 2004). In mice, 400 of the 10,000 genes examined did not have normal expression. It is interesting to note that these abnormalities were observed in placental cells but not in

the foetal part. This fact may explain the frequent malfunction of the placenta in clones (Fulka *et a*l., 2004).

Some observations suggest that early developmental arrest of cloned embryos may be caused by an immune-type rejection. Indeed, the trophoblasts of cloned cattle express MHC1 genes at variable rates and in a deregulated manner. An abnormally high number of CD3+ T lymphocytes has also been observed around endometrial cells in cows carrying cloned embryos. This could indicate the existence of atypical immune reactions (Ellis, 2004).

It is therefore likely that clones are more often epigenetically modified rather than carriers of gene mutations in the strict sense of the term (Smith and Murphy, 2004).

Some genome abnormalities, such as the length of the telomeres, disappear in the progeny of clones (Schiels and Jardine, 2003). Some hypermethylated sites in cloned mice remain hypermethylated in their progeny (Lane *et a*l., 2003). On a molecular level, it seems that not all the traces of cloning are completely erased by a normal reproductive cycle.

2 Domestic animal cloning applications: advantages and limitations

2.1 INTRODUCTION

In species of zootechnical interest, genetic progress depends on the effectiveness of reproductive techniques just as much as on the precision with which the phenotypic traits of individuals can be evaluated. Various reproductive techniques have been employed since the invention of livestock farming 10,000 years ago. Control of reproduction led to the first selections in domestic species. Artificial insemination and then embryo transfer have led to much more rapid genetic progress over the last few decades (see appendix).

Cloning, which avoids the uncertainties of reproduction, theoretically opens up possibilities for directing and accelerating genetic improvement in livestock. These theoretical schemes are based on the hypothesis that it is the progeny of clones and not the clones themselves that will be offered to consumers.

Despite its limited efficacy, the cloning technique is already applicable in animal husbandry, at least on a small scale. It appears that the major stumbling block to the application of cloning in farm animals is the cost of cloning breeding animals identified as having a high genetic value. It is widely accepted that the costs of cloning will fall very significantly in the next five years. Over this same period, the use of cloned breeding cattle is likely to increase exponentially. At least it is on these hypotheses that certain selection companies are basing their development forecasts (Faber *et a*l., 2004).

The selection of cattle in France is highly organised. The current system is described in the appendix. All the indications are, therefore, that the development of blood lines derived from cloned breeding animals would not be conducted in an anarchic or uncontrolled manner.

The use of cloning is inevitably accompanied by a reduction in genetic diversity. This brings theoretical risks, in addition to those related to artificial insemination and embryo transfer. Defects in the current selection system could help to amplify the intrinsic risks of cloning. It is therefore necessary to define the conditions for use of cloning likely to lead to genuine genetic progress without an excessive reduction in genetic diversity.

2.2 CLONING APPLICATIONS

2.2.1 Potential applications of fully mastered animal cloning

This section aims to provide an inventory of potential cloning applications, which is as exhaustive as possible. The type of cloning to be considered as a priority is cloning using somatic cells from individuals that may have demonstrated a serious of aptitudes of interest in their lifetime, liable to motivate potential users. There are still too many uncertainties with respect to the genetic value of an embryo as a potential nucleus donor. It is, of course, possible to envisage cloning of an embryo, following typing and selection, for a whole series of known genes and/or an estimated genetic value via the QTL theory (research in this field is developing fast). But this is a strategic fallback position, which one would be forced into, particularly if it were to prove impossible to very significantly reduce the frequency of phenotypic defects among the products of somatic cloning.

We are therefore looking forward to an ideal future, when the current technical and scientific problems relative to cloning will have been fully mastered. We would then have individuals in which the nuclear DNA was an exact copy of that of the initial individual (donor). Obviously, the production cost of obtaining these clones will depend on the elimination rates observed at each stage of the cloning operation. In addition, in this hypothesis, we are not taking into account the fact that pre-existing somatic mutations in donors, related to aging for example, could be problematic.

This projection also takes into account future advances in knowledge of the natural genome (on the basis that a great deal of resources are being deployed in this area worldwide and that research is making rapid progress) and its manipulation via transgenesis. In concrete terms, therefore, we are not

expecting cloning to play a dominant role in genetic progress, which can be obtained using other reproductive techniques and molecular marking (selection assisted by markers).

The objective is more to determine the applications *of objective interest* to the world of animal husbandry *because no method other than cloning permits, or permits within a reasonable period of time, the same result to be obtained.* We can identify at least four areas of application.

a) *emergency cloning*

- An animal of interest in selection programmes (a bull with very favourable test results for artificial insemination, for example) may accidentally become incapable of fulfilling its role as a sire. It can be cloned to avoid a loss of genetic progress.

- A non-breeding animal (for example a show jumping gelding) may be of potential interest for reproduction. Cloning the animal will promote genetic progress.

- An animal of interest to the breeder may (for whatever reason) have to be retired from use. It may be desirable to replace it with a "copy", which will itself serve as a breeding animal.

- An embryo cultivated for transplantation into an adoptive female may be the source of nucleus donor cells. Indeed, it is possible to take a few cells from an embryo without compromising its development. Embryos obtained by cloning from these cells may produce a high number of cells, sufficient to permit genetic typing of the original embryo. This approach enables a posteriori correlation between genetic markers and the zootechnical performance of the animal.

Obviously, these are applications in which cloning opens up totally new possibilities.

b) *cloning for genetic dissemination*

It would be preferable that some herds (beef cattle, dairy cattle partially) employing natural service methods, could benefit from the advantages of this type of technique while immediately having access to the better genetic quality of the breeding animals used in artificial insemination. In this case, some of these sires could be cloned and directed to natural service. The economy of these herds would therefore be improved, while at the same time stimulating genetic progress in the long term. At this stage of the argument, it is necessary to give a minimum of attention to maintaining genetic variability. To do this, a lot of different clones would have to be circulated, if possible selected from amongst those least represented in herds.

Obviously, it is also possible to envisage cloning females having demonstrated a whole series of interesting zootechnical capacities throughout their careers.

In this type of application, cloning simply makes it possible to obtain a certain desired genetic quality, more rapidly than using conventional reproductive methods.

c) *cloning for production*

Here, cloning is a basic component of the production system since it brings immediate economic benefits to the farmer employing it: in short, cloning makes it possible to *invent new production systems*. The following examples illustrate this type of possibility.

With respect to dairy cattle, the interested farmer obtains several clones of cows with a very well balanced genetic profile (good production but no fertility or mastitis problems), which, as a result, have a very good longevity (reduction in depreciation costs).

Farmers usually ensure reproduction of these animals by crossbreeding (use of calves), except when replacement with other more recent clones is envisaged. In this situation, the balance of the system is related to the production cost of the clones. In this case, there is no impact on genetic progress in pure breeds but the farmer exploits trait-combining genetics to maximise his immediate profit.

In the case of beef cattle, a "clone breeders" and fattening house association could lead to economically interesting labels. Indeed, the known performances of the original donor could and should include not only the slaughter performance but also detailed characteristics of the meat quality (these are of partially genetic origin). Consumers are increasingly demanding in terms of meat quality

while conventional selection methods are either too inaccurate or too costly (slaughter of progeny). Cloning would provide a neat solution to this problem.

In both cases, genetics are being exploited combining traits which are very rare because they are difficult to obtain simultaneously in a short time using other techniques, even with the help of molecular genetics.

d) *cloning for the protection of variability*
Cloning leads to the accelerated destruction of genetic variability if the individuals that are cloned are very commonplace and hence closely related on average with the existing population. However, it leads to a strengthening of genetic variability if it concerns rare endangered lines and, in a way, strongly increases their prolificacy. Obviously, the technique is fundamentally neutral and only certain conditions and prospective applications can have a detrimental effect.

Selected populations
Cloning females which are of interest but not very well represented within herds increases the frequency of their genotypes. This leads to a rebalancing of the gene pool and thus has a favourable impact on the possibilities of genetic progress in the long term *(cf.* known theoretical results in population genetics).

Preserved populations
Most of the time, the genomes of initial females are almost entirely lost since selection systems focus primarily on males. Prolonging their genetic life through the use of cloning would make it possible to build up large stocks of female gametes, which could be regularly drawn upon. The result of this would be a reduction in genetic drift and consanguinity in populations. An extreme application of the technique leads to the immortalisation of populations, with each retired individual being replaced by its own clone.

In these two types of populations, if financial resources allowed it, everything would point to supplementation of frozen semen stores with frozen cell stores with a view to potential cloning. It should be noted again here that cloning is the only technique that would enable the rapid resurrection of an extinct population.

2.2.2 Application conditions

The potential applications are therefore highly diversified and interesting. It must be stressed that the interest is *intrinsic*, and in particular that cloning is not presented as an alternative method for the creation of genetic progress. In this area, it is necessary to take into account present and future advances in molecular genetics, which will make selection more efficient and less costly. On the contrary, knowledge of the genotypes of clones for a certain number of genes of interest, in addition to their performance and selection index, will make it possible to better target those individuals worthy of cloning and hence reinforce the cloning application niches.

Full-scale transposition on the ground demands that the technique be fully perfected (this is not currently the case since the average success rate is still too low and too irregular) and that the clone purchase price be acceptable to the user (which will very probably depend on the application envisaged).

2.3 ECONOMIC ASPECTS OF CLONING

The cloning applications outlined above are all closely linked to economic considerations. The commercial applications of cloning livestock will essentially remain limited as long as the technique is not sufficiently reliable. It must continue to be the subject of in-depth research, both to resolve fundamental biology problems and to improve the cloning method itself. All the pointers indicate that improvements in the technique are possible and that this will lead to a substantial reduction in the production cost of clones intended to improve livestock production (Farber *et al.,* 2004).

At present, the costs of cloning in cattle, all breeds combined, are as follows. The current production cost of a cloned calf is around $20,000 and that of a cloned cow $170,000. The use of semen from a

cloned bull could lead to an annual profit of 1 million dollars (Powell, 2003). These prices should be compared with those of animals selected for conventional reproduction. A selected cow costs $2000 to $20,000 depending on the breed and a bull $3000 to $30,000. Prospective studies indicate that, if fully mastered, the cattle cloning technique would enable an additional profit of around $1000 per calf to be obtained in comparison with conventional artificial insemination. This is likely to gradually become a reality in the next five years and beyond. Indeed it is postulated that the price of a cloned calf could fall steadily as the technique improves, reaching a figure of $1000 in 2010 (Farber *et al.,* 2004).

2.4 VALUE OF CLONING TO SAVE ENDANGERED SPECIES OR BREEDS

For certain species not used for zootechnical purposes, living wild or semi-wild in the natural environment, the risks of extinction are real and often related to the consequences of pollution, hunting or over-farming of natural stocks on the balance of ecosystems. The disappearance of these populations therefore leads to a deterioration in genetic diversity and the loss of valuable genetic traits. For example, 30% of cattle breeds are not regularly farmed and are therefore threatened with extinction. These breeds can be preserved in the form of living animals and also in the form of frozen semen, embryos or oocytes.

In this context, cloning has been presented as a technology capable of contributing to protection of endangered species or breeds. The genome of animals can also be preserved in the form of frozen somatic cells, subsequently used to regenerate living animals using cloning techniques. In practice, fibroblast cells can be taken from the ears of the animals, cultivated, then stored in liquid nitrogen. Standardised conditions have been defined to establish and store cell lines in a reliable manner (Bousquet and Blondin, 2004). These types of collection have already been set up, for example at the Center for Reproduction of Endangered Species in San Diego (USA).

The use of this approach should be considered in two different situations: (i) firstly, the conservation of genetically important/interesting individuals or breeds within a species that does not present any conservation problems (ii) secondly, in the event of an endangered species. The first situation covers, in particular, breeds of agronomic interest. In this case, preservation of genomes in the form of frozen nucleus donor cells, which is recommended by the FAO (FAO/WHO, 2003), can be applied to the conservation of all valuable animals: animals with exceptional genetic traits, animals producing little semen, animals which have had accidents, transgenic animals and endangered animals. However, certain precautions must be taken to ensure that the biological material produced by cloning brings a positive contribution to the welfare of the population that one is trying to conserve. Indeed, if nuclear reprogramming is inadequate and leads to phenotypic abnormalities, the risk of ultimately producing cloned individuals that are counter-productive in terms of welfare is real. Working with the progeny of cloned sires should therefore be considered.

In the case of endangered species, it will therefore usually be necessary to use inter-species cloning. There is little chance that the oocytes used to receive nuclei derived from somatic cells can be obtained from the endangered species itself, meaning that it is necessary to turn to another closely related species. Thus cloning of the Gaur *(Bos gaurus)* has been performed using enucleated oocytes taken from cows *(Bos taurus)*. The hybrids resulting from this type of inter-specific cloning are of interest from the point of view of research but cannot always represent a genuine solution to save an endangered species.

3 Risks related to cloning: what are the repercussions in terms of animal health from a physiological, pathological and behavioural viewpoint?

3.1 INTRODUCTION

The number of living ruminants over the age of six months and obtained by cloning was less than a thousand worldwide at the end of 2003. In 2004, most of these animals are under five years old. These animals are the result of several tens of thousands of attempts to implant cloned embryos, since 5 to 10% of these produce living calves at the end of the gestation period, almost a third of which die during the first six months after their birth.

The health problems of cloned individuals are thus particularly marked during the intrauterine development stage, then during the first six months after birth. In addition, since cloned elite breeding animals are concerned, it is necessary to consider the health of their progeny (cloned or otherwise) and their potential impact in the animal populations where they are to be used. This concerns genetic diversity, propagation of factors leading to genetic susceptibility to various acquired diseases (microbial or parasitic transmissible diseases, metabolic diseases, etc.) or genetic diseases. It is therefore essential to understand the genetic or epigenetic nature of the abnormalities observed, along with their transgenerational impact.

Amongst the prenatal development and postnatal health problems described in cloned animals, a certain number have already been reported following the use of other reproductive techniques (in vitro fertilisation and embryo transfer, for example). This aspect must not be neglected, in order to understand and, if possible, control the impact of the abnormalities observed, on the level of both individuals and whole populations.

3.2 STATE OF HEALTH AND DISEASES IN CLONED ANIMALS AND THEIR PROGENY

3.2.1 Monofactorial and multifactorial diseases

In terms of animal diseases, a distinction is traditionally made between monofactorial diseases caused by a single causal factor (which may be a transmissible agent, a gene, a toxic substance, etc.) and multifactorial diseases caused by a combination of factors, usually in particular circumstances (agent with a weak intrinsic pathogenic potential developing in a weakened animal at a particular time in its life).

This classification applies, for example, to two viral infections: foot-and-mouth disease (monofactorial disease) and neonatal calf gastroenteritis following rotavirus infection (multifactorial disease).

In the same way, a genetic monofactorial factor is recognised in numerous conditions affecting domestic animals (thus almost 400 genetic diseases are recognised in cattle - M.I.C. 2000, OMIA).

Things get a little more complicated when it comes to genetic resistance to certain diseases. This more often involves resilience rather than true resistance, particularly in the area of parasitic diseases. Their genetic factor is often polygenic and could be explored by investigation of "Quantitative trait Loci" (Q.T.L.), but simpler determining factors can also be found, such as those involved in resistance to scrapie (TSSE).

In addition to disease (mono- or multifactorial), there are numerous situations in which there is cohabitation between a microbial agent and an animal host without any associated illness (adult cattle carrying *E. coli* 0157, H7, VTEC) or with only sporadic signs *(Listeria monocytogenes* and in sheep) but with public health consequences as a result of human contamination, notably through food products.

Finally, it should be stressed that in numerous pathological processes, in addition to the development of a disease in an individual, there is an induced risk for a variable proportion of the animal population (species, breed) to which the individual belongs, including through successive generations in the case of genetic diseases.

In order to analyse the risks that could be specifically associated with the health of animals produced by cloning, a distinction can first of all be made between risks associated with transmissible multifactorial or monofactorial diseases and those associated with a monofactorial genetic support.

3.2.2 Transmissible monofactorial diseases, multifactorial diseases and healthy carriers

In monofactorial transmissible diseases (MT) or multifactorial diseases (MF), the genetic component of susceptibility is not very significant (MT) or not fully understood (certain MT and MF). However, the implementation of genetic selection to improve production performance (quantity of milk, growth rate, meat quality) in certain species or breeds (dairy cows, for example) within intensive production systems has coincided with an increase in the incidence of enzootic multifactorial diseases affecting the locomotor system, the udder and the respiratory tract. These observations, made over a long period of time (several tens of years) have not been the subject of analytical studies which would have enabled the respective contribution of various factors (diet, living conditions, genetics, etc.) to be measured. The consequences of these conditions are very considerable (mortality, morbidity, costs, quality and quantity of products) since they represent the most common reasons for health intervention. A “loss of hardiness” related to the development of rational methods to improve productivity is widely cited, without this argument being really backed up. The same types of comments and questions (unanswered) exist with respect to healthy carriers (without any associated disease in the animal) of numerous mainly bacterial pathogens *(Listeri*a, *Salmonell*a, etc.) the consequences of which are well known in terms of public health.

All in all, for both healthy carriers and multifactorial diseases, there is a lack of knowledge relative to the general determining factors (causes, mechanisms, consequences) of the interactions between the pathogen and the target animal. In this context, particularly as far as the genetic influence is concerned, assessment of the consequences of the use of cloned animals in existing genetic improvement systems can only be based on considerable research efforts and studies on target species in experimental or production conditions, including a sufficiently representative number of animals and for significant durations (several generations).

Before performing cloning operations, it is therefore important to assess the health risks related to the state of health of the donor animals and, possibly, the recipient.

3.2.3 Genetic diseases

Paradoxically, this is a category of diseases which are well known, on both an individual and populational level, for which there exists real experience in terms of detection and emergence control. We will illustrate the experience acquired in cattle and what this can contribute in terms of cloned breeding animals.

Although more than 400 genetic abnormalities have been identified for all breeds on a worldwide level, the global prevalence of clinically expressed abnormalities is probably less then one per thousand. However, it must not be forgotten that the frequency of gene carriers can increase very fast and reach high levels (in 1992, in the United States, one Holstein calf in 200 was affected by BLAD - Bovine Leukocyte Adhesion Deficiency). In France, up until the start of the 1990s, the number of abnormalities having been the subject of research and/or control programmes was very limited (Arthrogryposis and Palatoschisis syndrome in the Charolais breed, polydactylism in Normande cattle). Over the course of the 1990s, three new diseases successively emerged in France: BLAD the frequency of which peaked in 1992, achondroplasia and CVM (Complex Vertebral Malformation) identified in France and Denmark respectively in 1999. These three abnormalities are identified in Holsteins. BLAD and CVM are autosomal recessive monogenetic diseases (like 60% of inherited bovine abnormalities). Achondroplasia is more complex and probably linked to a dominant major gene with incomplete penetrance (Ducos *et a*l. 2002, Hagemoser *et a*l. 1983, Agerholm *et a*l. 2001).

The appearance of multiple genetic abnormalities within a single Holstein dairy breed (or Prim'Holstein) raised the problem of their emergence conditions. These are at least partially related to the increase in consanguinity[1] in the majority of dairy breeds since this has increased by 1% per generation since 1970. Hence, in the Holstein breed, consanguinity increased from 0.5% in 1986 to 2.5% after 2001. This is due to the selection policy applied for the last twenty years, with a significant reduction in the number of bull sires and their very unbalanced use, which leads to significant bottle necks (in the Holstein breed, two founding bulls were carriers of BLAD and CMV).

Two quantitative aspects illustrate the importance of this phenomenon. In France, for a population of three million Holstein cows, there are 56 sires. For a recessive gene with a frequency of p = 0.01, the probability of the appearance of a genetic defect is 26 times higher for a mating with a consanguinity coefficient of 0.25 (parent x descendant mating) than for mating with a consanguinity of 0 (individuals produced by unrelated breeding animals).

3.2.4 Development of clones and problems identified

There is a very marked mortality among cloned embryos and a very high postnatal mortality rate, which is particularly acutely observed in sheep and cattle and much less so in goats and pigs. In this respect, it is necessary to note the very high frequency of hydramnios and hydrallantois observed in foetuses, both abnormalities that are extremely rare in ruminant populations.

The abnormalities are often related to placental abnormalities, which could be the cause of large offspring syndrome (LOS) in cattle and sheep. The data available are not very numerous and quite often inaccurate.

Cloned animals may be more sensitive to certain infectious diseases. They may therefore theoretically contribute to an increase in the frequency of certain diseases, independently of their genetic traits.

The reduction in genetic diversity resulting from cloning may also disseminate the genomes of animals more sensitive to certain diseases. Cloning may therefore ultimately, and in an unpredictable manner, contribute to a deterioration in the state of health of certain animal populations.

Conversely, cloning may pass on genomes making animals more resistant to a given disease. This may occur unbeknown to breeders or, in contrast, be a deliberate process if such a genome has been identified.

3.3 REPERCUSSIONS OF CLONING ON LIVESTOCK WELFARE

3.3.1 Context

The consideration of welfare criteria in the farming of species of commercial interest over the last twenty years has been a constant element, which is becoming an increasingly important factor in the assessment of new breeding techniques. The ethical principal laid down in the Treaty of Amsterdam considers that we have a moral obligation to animals, which prohibits us from causing them suffering, even if these actions are beneficial to humans. The general welfare rules stipulated can be grouped into the following five areas: preventing hunger and thirst, discomfort, pain, injury and diseases, fear and anxiety and at the same time permitting normal behaviour. Hence, welfare covers not only the health and physical condition of animals, but also their mental state and their capacity to withstand unfavourable fluctuations in their environment. For around ten years, animal welfare considerations have been applied to new animal breeding techniques. In this context, it was logical that animal cloning should also be evaluated in terms of its repercussions on the welfare of cloned individuals. This question was the subject of two reports, in 1998 and 2004, drawn up by the FAWC (Farm Animal Welfare Council, UK).

[1] The consanguinity coefficient is the probability that, on a given locus, the gene transmitted by the father and the gene transmitted by the mother are the replication of the same ancestor gene.

3.3.2 Impact on cloned animal welfare

Without condemning cloning out of hand on the basis of non-compliance with the rules of animal welfare, animal welfare experts nonetheless consider that several of the operations applied during cloning pose genuine welfare problems:

1. The first objection concerns “large offspring syndrome”. It is not uncommon for abnormal foetal development to be observed following in vitro fertilisation and nuclear transfer during which the embryo is kept in an in vitro culture medium before being transferred to the adoptive mother. This leads to the production of offspring of an abnormally large size, which poses problems to both the mother and newborn at the time of birth.

2. The second criticism concerns the state of health of cloned animals. As has been indicated above, three situations are observed at the current time: (i) clones which present serious abnormalities and die during gestation, (ii) clones which present reversible disorders but which survive after birth, (iii) normal clones. In this context, current practices raise issues with respect to animal welfare rules insofar as it can be stated that these abnormalities are caused by the technique used (cloning) and do not constitute a rare event as is observed in conventional breeding methods.

3. The third criticism of cloning concerns the risk of loss of genetic diversity. In the absence of any genetic control of the individuals obtained by cloning, there is a risk of impoverishment of genetic diversity, which could in turn foster the dissemination of genetic abnormalities, the emergence of new susceptibilities to certain transmissible diseases or to stress in farming conditions.

As is often the case in debates on the subject of farm animal welfare, criticisms of current practices are the subject of additional research and technical solutions that are usually acceptable are proposed. However, in the majority of cases, this research is followed by significant changes in farming methods. Cloning must be evaluated in the same way. It can be reasonably expected that the problems raised in points 1 and 3 could be better controlled and hence become acceptable in terms of welfare rules. Responses to the criticisms concerning the health of cloned animals will be more difficult to provide insofar as the abnormalities observed appear to be inherent to the cloning technique. It is nonetheless entirely conceivable that improvements made to cloning techniques will make it possible to reduce the detrimental effects currently observed.

4 What is the impact on the genetics of the species concerned?

4.1 IMPACT OF CLONING ON GENOMES

By definition, the effect of cloning is to multiply the genome of the animal from which the clone originates into as many copies as there are progeny. This must be considered for both "first generation" clones and for subsequent generations, produced by serial sub-cloning from a primary clone. Cloning performed by nuclear transfer from somatic cells taken from an old breeding animal therefore has the same effects as prolongation of the breeding animal's life, making it possible to obtain a greater number of gametes from him/her for example. From this angle, cloning is more advantageous in females than males since the number of oocytes liable to be produced is incomparably smaller than the number of spermatozoa produced by the male. This is further accentuated by the greater difficulty in preserving female gametes, in certain species at least.

In most species in which clones have been produced, animals have survived to reproductive age and, in practically all cases, they have been able to produce apparently normal offspring following mating with normal sexual partners. This observation is encouraging when compared to the significant disease affecting the animals born of manipulated embryos since it seems to confirm that meiosis acts as a "genetic abnormality filter", only letting normal gametes through. This observation confirms previous observations, already made in mice.

Preliminary experiments have been performed with the aim of demonstrating the integrity of DNA taken from the cells of cloned animals and its similarity with the donor's DNA. These experiments, based on the identification of any polymorphisms in the size of microsatellite PCR amplification products (Simple Sequence Length Polymorphism or SSLP) have not detected any substantial modifications between the two genomes. These experiments are preliminary, relatively crude and certainly inadequate to detect subtle, occasional and probably rare modifications. Other results, based on sequencing of large DNA fragments analysed in terms of Single Nucleotide Polymorphism (SNP) are necessary.

The dominant mutations and chromosomal abnormalities which occur in the somatic cells of an organism during development have little impact on the organism in question since they are generally incompatible with survival of the cell and are therefore rapidly eliminated. When they are present in somatic cells removed to perform nuclear transfer or when they occur during in vitro culturing of explanted cells, they generally lead to failure and do not produce a viable embryo. It is also considered that these mutations and other chromosomal abnormalities are responsible for a proportion of the failures observed in nuclear transfer cloning. This leads to the assumption that, since it is very strongly counter-selected, this type of abnormality reduces the risk of cloned animals inheriting damaged genetic hereditary material.

Recessive mutations increase in number with cell generations and each daughter diploid cell inherits mutations present in the parent cell, to which it adds others. These mutations are naturally eliminated because they are confined within the somatic cells and do not therefore have any genetic future, or because they are not represented in the gametes involved in production of generation G + 1. From this point of view, it can be considered that here meiosis is, once again, a "genome-cleaning" mechanism. Cloning short-circuits the effects of this mechanism and thus enables mutations to accumulate. To illustrate this aspect, it can be noted that "sub-cloning" or serial cloning appears to be possible a limited number of times only (three times in cattle and seven in mice) and one of the explanations generally put forward to account for this limitation is that the mutations accumulated in the somatic cells ultimately prevent the production of a viable embryo. It is nonetheless difficult to make a distinction between the effects of conventional mutations and epimutations that affect the expression of certain genes but not their structure.

Mutations occurring in mitochondrial DNA (mtDNA) represent a potential risk for cloned animals. Mitochondrial DNA is an important structure for the correct function of the cell metabolism and its integrity is essential. This substance is inherited from the mother only since the sole mtDNA molecule present in the spermatozoon is lost at the time of fertilisation. Because it is haploid, mtDNA is also a structure that mutates readily in the somatic cells, leading to relatively high rates of heteroplasmy, in

other words a mixture of mitochondria with DNA of different structures. When a cell nucleus is removed for transfer into an oocyte, contamination of the oocyte in question by mutant mtDNA molecules is possible. Even if these accidentally transferred molecules are in the minority to begin with, it has been shown that, with time, they can totally or predominantly replace the mtDNA molecules of the host cell and hence lead, at least in theory, to the observation of a pathological condition. This potential risk primarily concerns female clones and their progeny since there is a “bottle-neck effect” in the transmission of mtDNA from mother to daughter. Indeed male clones only pass down nuclear DNA to their progeny.

Epigenetic modifications are commonly observed in the genome of clones, concerning methylation of DNA and histone post-translational modifications. These epigenetic modifications, along with the increase in telomere size observed in certain cloned animal cells, appear to be largely reversible when the genome goes through the haploid phase (gametogenesis). Consequently, they only represent a very low potential risk for the progeny of clones.

Cloning of male breeding animals can also, theoretically at least, have effects on the frequency of alleles in the population of animals belong to the same breed when the semen of the sire in question is used for artificial insemination. Indeed, the fact that a sire is used for longer and more frequently mathematically modifies the frequency of alleles in the breed in question. These effects are mild if the number of clones remains low but are added to the effects of artificial insemination.

4.2 IMPACT OF CLONING ON REDUCTION IN THE GENETIC DIVERSITY OF FARMED LIVESTOCK POPULATIONS

Conventional genetic selection, performed by means of artificial insemination, is already being accompanied by a reduction in the genetic diversity among the population. Cloning can only amplify these effects if certain precautions are not taken (Cf. above). This reduction in genetic diversity could have several negative repercussions. It could, for example, make certain herds more sensitive to rare pathogenic agents. Such a situation could lead to unexpected breeding problems. What’s more, the possibility that even the most rational application of cloning may, in theory, induce these types of effect cannot be totally excluded. The low but not nil probability that such a scenario could become a reality can be unpredictably increased by changes in the conditions in which the animals are farmed. For example, climate changes could encourage the preferential emergence of certain diseases in herds composed of a high number of clone progeny, too genetically close to one another.

Theoretical studies have defined the boundaries that users of cloning techniques should not cross (Colleau, 1993; Colleau *et al*.,1998; Wooliams and Wilmut, 1999).

Quantitative genetic testing currently offers effective solutions for the genetic management of selected populations (Bijma *et al*., 2002, Colleau *et a*l., 2004a,b). These solutions would also be applicable to populations in which clones were used and enable suitable management of such herds. Hence the use of clones with very common pedigrees is not really recommended whereas the use of clones with rare pedigrees is strongly recommended, which, in practice, will help increase genetic variability.

A few examples can illustrate these points. Conventional genetic selection has made it possible to very significantly increase the milk production of cows. This is accompanied by harmful side effects which tend to increase as selection to improve milk production progresses.

The most productive cows demonstrate a steadily falling fertility rate. This is a complex phenomenon that has not yet been explained. The reduction in genetic diversity accompanying this selection may have co-selected genes favourable to lactation and others unfavourable to reproduction. It is also conceivable that the two biological functions involved, i.e. gestation and lactation, which naturally compete with one another metabolically, are placed in an increasingly incompatible situation following intense selection.

In the same way, it has been established that the frequency of udder infections is higher in cows that are high milk producers.

During the first few weeks following calving, dairy cows are subjected to a metabolic imbalance forcing the animal to draw intensively on its own reserves. This phenomenon is often accompanied by a

variety of metabolic diseases (hepatic steatosis, acetonaemy, greater sensitivity to milk fever), which it is difficult to control.

It is logical to believe that imprudent use of breeding animals obtained by cloning could help to accentuate these trends. Conversely, the introduction of the genetic material of breeding animals validated by a breeding career could significantly contribute to controlling the excesses of conventional selection methods. Indeed, there are individuals within herds which are both good milk producers and animals that do not suffer from the metabolic disorders cited above. The number of these animals is too small to be able to use them as breeding animals capable of having a significant genetic impact on herds. If it were technically well mastered and carefully targeted, cloning could lead to dissemination of these favourable “deviants” and help resolve the problem (cf. 2.2.1 b) and c)).

If ever there was a field where the principle of precaution should be observed prior to any application, it is that of cloning, due to the marked genome modifications (epigenetic mainly) frequently observed with the current technique and which are linked to the very high loss and/or abnormality rate during development.

As long as research has not managed to better control these phenomena, it is preferable not to consider applications in which the animal resulting from cloning is involved in reproduction, in order to avoid any risk of durably altering the genome of the entire population after several generations. It is obviously urgent to define on the basis of which criteria and which methodology a clone may be declared suitable or otherwise for reproduction. Very clearly, this theme requires in-depth study by researchers. Whatever the case, the “production” cloning described earlier in this report is free of this concern. It is probably in this field that a rapid application of cloning would not include any poorly grasped risks.

5 State of knowledge relative to the quality and safety of food products derived from cloned animals

The two main food products derived from cloned cattle – milk and meat – have already been subjected to tests designed to identify any potential problems related to their digestibility, nutritional properties, toxicity, allergenicity and mutagenicity. The tests performed currently and which are described in this chapter essentially concern the clones themselves. These data have been obtained from a limited number of animals. It is also important to note that the products that are liable to be offered to consumers are derived exclusively from the progeny of these clones and not from the clones themselves. Tests must therefore be applied primarily to the progeny of clones, even though all the indications are that the defects of clones are greatly reduced or even non-existent in their progeny.

5.1 COMPOSITION OF MILK AND MEAT

The composition of milk has been examined independently by at least three groups (Seamark, 2003; Walsh *et al*., 2004; Norman and Walsh, 2004) in a significant number of animals: 15 and 13 cows respectively. No significant differences have been observed relative to the following parameters: total solid quantity, minerals, proteins, fats, lactose, pH, SCC (somatic cell counts), ADV (acid degree value). A more detailed examination has also revealed that the proportion of the various milk proteins (caseins and lactoserum proteins), along with the relative concentration of the various fatty acids, is identical in cows obtained by cloning and in control animals. The first experiments conducted with the progeny of clones confirm these conclusions.

Milk from cows, goats and sheep contains more than a million different substances (Jenness 1988), the presence of which – and hence the composition of the milk – varies depending on the breed of cow, the lactation phase, the age of the cow, the interval between milkings, the atmospheric temperature, diseases and the season of the year (Walstra and Jennes, 1984; Kaufman and Hagemeister, 1987). The detection of differences between clones and uncloned cows would require analysis of all these effects.

The overall composition of the nine parts of the carcass of cloned cows has also been evaluated on the basis of the following parameters: water, proteins, fats, carbohydrates, ash and cholesterol (Takahashi and Ito, 2004). No difference has been observed between the cloned animals and the controls.

Tian *et al.* (2005) analysed more than 100 parameters relative to the quality of the meat from 2 cattle and the composition of the milk from 4 cloned dairy cows. They also conducted histological tests on the organs of the 2 cattle at autopsy. The results do not reveal any significant difference between the cloned animals and the controls, nor any histological abnormalities in the organs examined (liver, kidney, lung, heart, spleen, adrenal glands and thyroid). This study, conducted in a limited number of animals and on clones produced by a single genetic source, needs to be extended to a large number of cloned animals of different genetic origins.

5.2 DIGESTIBILITY

In vitro tests using gastric and intestinal extracts have been used to evaluate the digestibility of meat homogenates from cloned cows and control animals. These tests have not revealed any difference in digestibility between the two groups of animals (Seamark, 2003; Takahashi and Ito, 2004).

5.3 TOXICITY AND NUTRITIONAL PROPERTIES

Rats were fed for 14 weeks with freeze-dried meat from cloned cows and control animals. The conventional parameters used to detect food toxicity and their nutritional properties were examined in these animals: general condition, behaviour, food consumption, fatality, growth, reproduction, lactation, along with 29 physiological parameters including, among other things, analysis of blood and urine and measurement of 9 reflex reactions. In addition, the animals were autopsied and the main organs were subjected to histological examinations (Seamark, 2003;Takahashi and Ito, 2004). These tests did not reveal any abnormalities in rats having consumed products derived from cloned animals.

A similar study conducted in rats fed with milk or meat from cloned cows confirms all of these conclusions (Tomé *et a*l., 2004).

5.4 ALLERGENICITY

In order to avoid the aspecific allergenic effects frequently observed in rats consuming meat, other allergenicity tests not involving the consumption of meat were performed. These consisted in sensitising rats with injections of meat extracts and then measuring the cutaneous reactions of animals locally exposed to meat extracts. These tests did not demonstrate any allergenic reaction specifically induced by meat extracts derived from cloned animals (Takahashi and Ito, 2004).

Measurement of immunoglobulin concentrations in rats fed with milk or meat derived from cloned or non-cloned cows demonstrated that the products derived from cloned animals did not induce any specific immune reaction. In rats fed with extracts of cloned or control animals, IgG, IgA and IgM antibodies were identified, but no IgE antibodies directed against the milk or meat. The immune reactions were similar in both batches of rats (Tomé *et al*., 2004).

5.5 MUTAGENICITY

The mutagenic properties of meat extracts from cloned or non-cloned cows were evaluated by the micronucleus test. No mutagenic effect was observed with products derived from the two groups of cows (Takahashi and Ito, 2004).

6 Conclusions

Animal cloning is a complex and still highly empirical technique, which can theoretically be employed to accelerate genetic progress.

Despite its still limited efficacy, the cloning technique could already be used to perpetuate high-value breeding animals within herds. It is therefore probable that cloned animals will soon enter conventional herds. It is important to note that the reproduction of breeding animals by cloning will for a long time be reserved for a few elite individuals. The clones will be obtained not to be consumed but to themselves produce animals which will be destined for human consumption. The physiological status of clones and the quality of their products are therefore less important from this point of view than those of their progeny in terms of food safety.

Observations made to date on cloned farm animals lead to contrasting conclusions. A significant proportion of them suffer from various temporary impairments to their health. These effects are the result of various and complex phenomena, which cannot yet be accurately described at present. However, once they are adult, animals born as a result of cloning are barely distinguishable from those born as a result of fertilisation. More in-depth studies conducted at a molecular level, particularly in mice but also in cattle, indicate that the few differences sometimes observed in clones have disappeared in their progeny.

From the point of view of food safety, the question is therefore raised as to whether these clones should be treated as conventional animals, as a new food or as belonging to an intermediate category.

Afssa (the French Food Safety Agency) wanted to review the state of knowledge in the field of animal cloning and to assess the risks relative to the consumption of products derived from cloned animals. In addition to food safety aspects, genetic aspects, as well as those related to genetic diversity, animal health and welfare, were also considered to be important factors to be taken into account in this assessment.

6.1 THE PHYSIOLOGICAL STATUS OF CLONED ANIMALS AND THEIR PROGENY

Examination of some 1500 cloned animals born worldwide demonstrated that a proportion of their abnormalities were essentially diverse and reproducible. It is remarkable that the very great majority of calves that survive the first few months after their birth without any damage have similar lives to those of animals obtained by fertilisation. Logically, it would be possible to consider that these clones – and particularly their progeny – are not different from animals obtained by conventional reproductive methods and that no particular examination is justified for their introduction into the human food chain. This is the position recommended by the FDA (Food and Drug Administration) in the United States (Powell, 2003).

However, the innovative character of clones and the abnormalities that some of them suffer, at least during an initial period of their life, suggest that it is necessary to carry out more in-depth assessments than those applied to animals obtained by fertilisation.

The physiological and zootechnical characteristics of clones and their progeny should be evaluated for at least two generations. It is probable that examination of the direct progeny of clones will be sufficient to ensure that they pose no specific physiological problems. The analyses to be developed should be based not only on conventional physiological tests but also on transcriptome analyses used as an indicator of the normal status of the animals.

Measurement of morphological, metabolic, endocrine and immune parameters, traditionally applied to conventional animals, ought to be conducted in the same way for clones and, in particular, their progeny. A non-exhaustive list of these parameters, but one which is representative of the physiological status of the animals, was outlined previously (part 1.4.1) and also by Rudenko *et a*l. (2004).

6.2 ANIMAL HEALTH

The apparent health of animals obtained by cloning is not normally any different from that of conventional animals. However, new clones obtained from primary clone cells in cows are more fragile than the progeny of clones obtained by sexual reproduction. In contrast, this phenomenon has not been observed in mice. This clearly indicates that cows obtained by cloning are not strictly identical to conventional animals. It has also been observed that mouse clones live, on average, 10% less time than their non-cloned counterparts. This is apparently due not to premature aging of the animals but to a greater sensitivity to conventional infectious diseases. An observation made in cattle suggests the same thing. Some clones suffer or die from rare or conventional diseases with an abnormally high frequency. These facts have been established on the basis of a very small number of animals, which prevents us from reaching any general conclusions, but which suggests that specific studies ought to be conducted in animals.

Additional research
Objective data relative to the evaluation of the health and immune status of cloned animals are not very numerous and are not always based on measurable or laboratory parameters. Studies need to be conducted to obtain additional information in these areas. For example, the following studies could be conducted to compare the health and immune statuses of cloned animals with those of conventional animals, and notably in comparison with donor animals.

- Document the health monitoring of cloned animals in comparison with a representative sample of conventional animals (more specifically donors or animals in the same environment as donors), taking into account serological profiles with respect to the main contaminants, vaccinate cloned animals against the main pathogens and compare the immune responses – both serological and linked to cell-mediated immunity – using cumulative response measurement parameters (Bruce *et a*l., 1999).

- Document any changes in immune functions in cloned animals (taking into account the specificities of the immune system of the species concerned), comparing the cell populations between cloned animals and a representative sample of the species concerned (including donors) or relative to average values if these are known, on the basis of a differential blood count, measuring the distributions of target cell populations by flow cytometry on peripheral blood mononuclear cells: T lymphocytes, including CD4/CD8, B lymphocytes, etc., conducting comparative measurement of myeloid and lymphoid line stem cells by bone marrow puncture.

- Attempt to establish correlations between cloning protocols and the type and frequency of abnormalities, the success of intrauterine development and postnatal health problems.

- Undertake the required pathophysiological studies concerning the intrauterine development of clones in order, particularly, to understand the determinism of LOS (large offspring syndrome) and that of hydramnios and/or hydrallantois, identifying the respective contributions of the two phenomena in intrauterine development problems.

Evaluation of animal health risks
Surveillance to detect any sensitivity of clones and their progeny to certain diseases therefore appears to be necessary to ensure the health of individual animals, but also to protect herds from the potential emergence of diseases spread more particularly by clones (cf. 3.2.4).

In terms of evaluating the animal health risks, the following measures should be implemented:
- evaluate the health of donor and recipient animals prior to cloning, along with the presence of the main pathogens, depending on the species concerned;

- conduct a comparative study of gastrointestinal and respiratory flora in cloned animals and a representative sample of the species concerned, including donors farmed in the same environment, including certain microbial agents such as Salmonella and *Campylobacter;*

- identify the key factors in postnatal mortality up to the age of 6 months (including laboratory tests, and particularly microbiological ones);

- systematically screen for known genetic markers of hereditary diseases in cloned animals *(c*f. 3.2.3);

- conduct constant health monitoring (until death, with systematic autopsy) of each cloned animal;

- use ad hoc protocols to observe the fate, from a health point of view, of several (at least four) generations derived from cloned animals.

6.3 IMPACT ON THE GENETICS OF THE SPECIES CONCERNED

The reduction in genetic diversity resulting from poorly controlled use of cloning could have various negative effects in the long term. The negative impact of conventional artificial insemination on genetic diversity has already been realised. Technical solutions to ensure healthy genetic management of herds already exist and are effective, as long as there is a genuine will to apply them. This is not always the case as often as it should be with conventional artificial insemination. More rigorous application of livestock selection methods is thus highly desirable with the use of cloned breeding animals. As a general rule, cloned breeding animals with a frequently represented pedigree should only be used moderately in order to avoid accelerating further still the reduction in genetic diversity. Conversely, favouring the use of cloned breeding animals with a rare pedigree could be recommended since this leads, in practice, to an increase in genetic diversity.

The examples cited in part 4.2 suggest that we should not automatically consider that the use of cloned breeding animals inevitably leads to a reduction in genetic diversity in herds. On the contrary, rational selection of breeding animals obtained by cloning may help to control the damaging effects of conventional selection methods. The success of the business is based on the rigorous selection of the breeding animals to be reproduced by cloning (genetic originality and good balance in zootechnical profile), but also on an in-depth analysis of the genetic heritage of the herds, which are then likely to benefit from an enrichment of their genetic diversity.

In addition, it is essential that the integrity of cloned genomes be as complete as possible (in particular without any epigenetic-type modifications). The diagnostic method that is still to be developed should be based not only on genetic markers associated with QTL of the most significant traits, but also on other non-specific markers, particularly in the regions of the genome where the genetic footprinting phenomena normally occur during development.

Experimental measurements of the genome expression of clone progeny could also be performed by means of systematic examination of their transcriptomes and their proteomes.

6.4 IMPACT OF CLONING ON THE WELFARE OF CLONED ANIMALS

According to the principle stipulated in the Treaty of Amsterdam, welfare covers not only the heath and physical condition of animals but also their mental state and their capacity to withstand unfavourable fluctuations in their environment. The consideration of welfare criteria in the farming of species of commercial interest over the last twenty years has been a constant element, which is becoming an increasingly important factor in the assessment of new breeding techniques. In this context, it is logical that animal cloning should also be evaluated in terms of its consequences on the welfare of cloned individuals.

The abnormalities and pathophysiological disorders observed in cloned animals are inherent to the cloning technique but it is entirely conceivable that improvements in these techniques will make it possible to reduce the effects currently observed. However, it will be necessary to continue to be vigilant in terms of the impact of cloning on animal welfare and to incorporate welfare criteria in programmes to monitor animals derived from cloning.

6.5 QUALITY OF FOOD PRODUCTS DERIVED FROM CLONES

The main two food products – milk and meat – have already been subjected to tests on their digestibility, nutritional properties, toxicity, allergenicity and mutagenicity. The results of these studies obtained on a limited number of cloned animals (not intended for human consumption) do not reveal any difference between the animals tested and controls.

However, these types of tests should be applied, for a period of time at least, to clones and, in particular, to those of their progeny intended to be included in farmed herds.

All these tests appear to be important to document the biological properties of clones and to define the criteria enabling their human consumption to be authorised or otherwise. It is not yet certain whether these measures are justified for more than just a transient period. Indeed, it is conceivable that the repeated absence of problems for a period of several years could make the majority of these tests pointless. Long-term monitoring nonetheless appears to be necessary in order to be able to identify the diseases that clones and their progeny could contract and to prevent their emergence.

6.6 CONCLUSIONS AND GENERAL RECOMMENDATIONS

The analysis of the risks and benefits of cloning presented in this report refers mainly to cattle. It is in this species that the use of cloning could have a significant and relatively rapid economic impact. However, it should be considered that the conclusions resulting from this analysis can be extrapolated to other species of farmed livestock.

The data acquired suggest that animals descended from clones, which are the only ones liable to be offered to consumers, can be treated in the same way as their equivalents produced using conventional reproductive methods. The tests that have long been applied to conventional animals for the marketing of their carcasses should therefore protect consumers from any risk.

It is nonetheless necessary to carry out more in-depth evaluations, based on the measurement of various biological parameters. Relevant observations have already been made, but their number is too limited to permit any generalisations. It is therefore necessary to accumulate data concerning animals directly derived from cloning and, especially, their progeny, for several generations.

The detrimental effects of cloning within a species are reproducible and observed in all laboratories where these methods are used. It should therefore be possible to consider that the conclusions drawn from observations using a sufficient number of animals can be generalised. This implies that risk assessment studies could be restricted to a limited number of experimental animals subjected to in-depth examination and not performed on each cloned animal. However, any significant change in the cloning method may justify, on a case by case basis, a few comparisons with clones obtained using the current methods.

Creation of herds specifically dedicated to study of clones and their progeny

The studies on clones in the various laboratories concerned are currently conducted on a case by case basis on a few animals. The preliminary results obtained are interesting insofar as they indicate the points that probably require more in-depth study.

Current practices do not permit any generalisation, particularly as concerns the potential effects of cloning in the long term. It is therefore highly desirable to have a sufficiently large number of clones obtained and studied simultaneously, and hence in comparable conditions, to which we can refer.

The creation and maintenance of such herds are relatively expensive operations. International cooperation would thus appear to be more than desirable in this area. Projects of this kind could benefit from financial assistance from the European Union, which has declared itself favourable to this type of study.

Creation of a surveillance committee

The studies of the various laboratories involved in assessment of the side effects of livestock cloning could benefit from monitoring by a cross-disciplinary committee. The evaluations of this committee could help avoid redundancies and gaps in research programmes studying the risks involved in cloning. The committee's recommendations could also serve as guarantees for the bodies providing financial support for this research.

Appendix
The genetic selection system in France

A.1 INTRODUCTION

The genetic improvement of livestock breeds is a longstanding practice and its development, which is dependent on scientific progress, has, for example, led, to the setting up of a highly organised regulatory and technical system for cattle breeds in France.

In order to understand the interest and conditions for application of cloning techniques, it is first of all necessary to conduct a detailed analysis of the methods and system in place, which play an essential role in current conditions and the results obtained with respect to the quality of products. The potential contribution and role of cloning will then be developed.

In France, the genetic improvement of cattle is based primarily on a system resulting from application of the law on livestock breeding adopted in 1966. A precise legislative framework defines the missions of the various professional bodies for the smooth running of this system, piloted and constantly adjusted within a consultative committee reporting to the Ministry of Agriculture: the “Commission Nationale d’Amélioration Génétique” or National Committee for Genetic Improvement (CNAG).

This national system, the aim of which is to conduct genetic evaluation of breeding animals and organise the selection of the best of these, is founded (i) on a system of zootechnical data collection and management and (ii) a protocol for the identification, evaluation and selection of male breeding animals.

A.2 ASSESSMENT OF GENETIC BREED IMPROVEMENT METHODS

A.2.1 The system for zootechnical data collection and management

This system is designed to ensure the homogeneous organisation of collection, validation, processing and transfer of all data to a central genetic evaluation site. These data concern the identification of animals, civil status, production levels, morphology or other functional aptitudes that are systematically monitored and can help in the definition of selection schemes.

In order to guarantee the homogeneous quality of the data collected throughout France, this system is based on a series of protocols and technical regulations, approved by the Ministry of Agriculture and supervised by the “Institut de l’Elevage” (Livestock Breeding Institute). On the ground, information is collected by technicians working for accredited and specialised bodies: either “Etablissements Départementaux de l’Elevage” or Regional Livestock Breeding Establishments (EDEs) (of which there are 80 in France) or “Organismes de Contrôle des Performances” (Performance Control Bodies or OCPs) (milk monitoring, growth monitoring) for production data.

Overall management of these operations is the responsibility of regionally competent bodies, EDEs, accredited to fulfil this mission by the Ministry of Agriculture. For certain data on morphology and other aptitudes, data collection is performed in a complementary manner by breed societies (UPRA) or selection units.

Data processing, which is conducted via the “Système d’Information Géographique” or Geographic Information System (SIG) is organised in the form of a database distributed between “Centres Régionaux Informatiques” or Regional IT Centres (CRI) and the “Centre de Traitement de l’Information Génétique” or Genetic Information Processing Centre (CTIG at INRA). Each local, breed or national body supplying raw data (recording of milk weights, animal weights, inseminations, scores, etc,) or compiled data (lactations, index, etc.) has access to managed, validated and centralised information.

A.2.2 The system for selection of breeding animals

Conduct of programmes
Animal insemination centres are accredited by the Ministry of Agriculture as implementation centres or semen production centres.

Programme implementation centres are charged with using semen from accredited or authorised bulls for insemination by the Ministry of Agriculture and with drawing up contracts with one or more selection units to guarantee the regular supply of breeders in the zone where they are the exclusive operators.

Semen production centres are responsible for conducting one or more selection programmes. These are then called selection units.

Selection units are generally unions of implementation centres, organised on a national or regional basis depending on the importance of the programmes and the breeds. They are entirely responsible for these programmes and own the bulls and their semen which they then sell back to member implementation centres or possibly to other selection units after the bulls have been approved for insemination. They usually entrust basic cooperatives with the material implementation of certain aspects of the programme, such as on-farm progeny testing and semen production. However, they always recruit bull dams themselves and carry out planned mating and recruitment of young bulls and management of individual testing or progeny testing stations (production of beef cattle or breeding cattle).

Bulls can only be approved if:
- the bulls have been tested in the context of an accredited selection programme,
- a selection index has been calculated and published on the basis of results obtained following progeny tests, for each aptitude,
- the indices are sufficiently precise,
- the values of the index confirm that they are breed improvers.

Today, 29 selection programmes, in 14 breeds, are accredited by the Ministry of Agriculture and guarantee the quality of genetic evaluation and genetic improvement that can be achieved. The standards for accreditation of programmes and bulls are periodically reviewed by the Ministry of Agriculture. Eight dairy breeds and 6 beef breeds have selection programmes including progeny testing (tables 1, 2 and 3).

The selection base
The selection base for the various cattle populations is founded on all the animals identified, the progeny of which are recorded and validated by the EDEs. These animals are subject to on-farm performance evaluation (milk evaluation and growth evaluation). It is on the basis of all these animals that progeny evaluation operations are conducted and bull dams are recruited.

Examination of tables 1, 2 and 3 reveals that France has a very high population of evaluated cattle for the main dairy breeds used.

Table 1: General "milk" programme statistics
(sources: SCEES (Central Statistics Department) of the Ministry of Agriculture [(1)]; IE-FCL [(2)]; UNCEIA [(3)])

Breeds	Number of cows	Number of AI per breed of male (1)	Number of females inseminated	Number of cows for Milk Evaluation	Number of bulls tested (2)	Progeny testing rate (1)/(2)
Prim'Holstein	2 338 000	2 287 464	2 541 603	1 946 011	614	3 726
Normande	532 000	423 318	447 920	283 177	154	2 749
Montbéliarde	654 000	465 277	595 848	380 403	162	2 872
Abondance	53 000	31 476	39 604	19 847	15	2 098
French Simmental	33 000	23 059	22 644	13 861	11	2 096
French Brown	24 300	20 045	22 221	15 936	8	2 506
Pie Rouge des Plaines	28 000	11 932	15 281	10 311	7	1 705
Tarentaise	14 600	8 947	9 824	7 757	14	639
TOTAL	**3 676 900**	**3 271 518**	**3 694 945**	**2 677 303**	**985**	**3 321**

AI: Artificial insemination
FAI: First artificial insemination
The number of cows (primiparous females) is estimated on the basis of the 2000 agricultural census and evolutions observed each year for the number of FAIs.
The number of females inseminated includes cows and heifers inseminated (respectively 73% and 27% of the total)

Table 2: General "meat" programme statistics
(sources: SCEES (Central Statistics Department) of the Ministry of Agriculture [(1)]; IE-FBC [(2)]; UNCEIA [(3)])

Breeds	Number of cows[1]	Number of AI per breed of male	Pure breed	Number of inseminated females	Number of cows for Cattle Growth Evaluation	Number of bulls tested		Number of progeny tested	
						Maternal Quality	Meat aptitude	Maternal Quality	Meat aptitude
Charolaise	1 666 000	489 996	222 468	229 928	247 276	35	0	19	19
Limousine	861 000	261 649	110 552	120 046	143 292	12	10	12	12
Blonde	441 000	163 257	116 970	120 532	98 758	10	5	7	16
INRA 95	(-)	53 310	(-)	(-)	(-)	(-)	8	(-)	8,5
TOTAL	**2 968 000**	**968 212**	**449 990**	**470 506**	**489 326**	**94**	**15**	**38**	**47**

AI: Artificial insemination
[1] : The number of cows is estimated on the basis of the 2000 agricultural census and evolutions observed each year for the number of FAIs.
[2] : Name of the performance testing protocol for "meat" programmes, conducted by the Institut de l'Elevage and France Bovin Croissance.

Table 3: Statistics for crossbreeds or "milk-meat" conversion breeds
(sources: SCEES (Central Statistics Department) of the Ministry of Agriculture [(1)]; IE-FBC [(2)]; UNCEIA [(3)])

Breeds	Number of cows[1]	Number of AI per breed of male	Pure breed	Number of inseminated females	Number of cows for Milk Evaluation	Number of cows for Cattle Growth Evaluation[2]	Number of bulls authorised for AI
Rouge des Prés	51 000	9 856	9 316	10 633	41	12 195	4
Salers	188 000	11 041	9 656	17 555	1 897	27 625	2
TOTAL	**239 000**	**20 897**	**18 972**	**28 188**	**1 938**	**39 820**	**6**

AI: Artificial insemination
FAI: First artificial insemination
[1] : The number of cows is estimated on the basis of the 2000 agricultural census and evolutions observed each year for the number of FAIs.
[2] : Name of the performance testing protocol for the "meat" programmes.

The situation is slightly different for beef breeds: despite an increase in the population of cattle tested, the numbers are not yet sufficient to be used as an exclusive progeny testing support. Adjustments to the on-farm performance evaluation protocol are currently being studied to this end. The population tested nonetheless serves as a reservoir for the recruitment of breeding animals, thanks in particular

to the on-farm genetic evaluation of breeding animals (IBOVAL). A quarter of the cows assessed are inseminated to improve the genetic quality of these herds and to ensure the genetic connection between herds.

Selection with respect to parents
Systematic identification of the best cows, careful selection of bull sires, the use of planned mating and embryo transfer make it possible to introduce the best breeding animals of their generation into selection systems.

Around 20% of embryo transfers have been performed in the context of collective genetic improvement schemes. In particular, embryo transfer (ET) makes it possible to create and manage cores of heifers with a very high genetic quality, destined to produce breeding animals which are therefore the sons of the best bulls and heifers, themselves the daughters of the best bulls. The shortening of the generation interval, made possible by increased selection pressure in the dams of future bulls (ET and, particularly, OPU-IVF – or ovum pick up-in vitro fertilisation), and by the certainty of obtaining at least one male calf for each female of interest, guarantees more rapid genetic improvement. This process supplies 50% of the dairy schemes for the major breeds.

The rest of the recruitments are made conventionally through matings between bull sires and bull dams. The latter are selected on the basis of extremely stringent criteria, placing them amongst the top 1 to 2% evaluated breeding animals of the breed. These matings are obviously conducted in the context of dairy schemes, but also (and here France is in an innovative position worldwide) in the context of collective schemes applied to beef breeds.

A marker-assisted selection programme has been in place since 2000 in Holstein, Normande and Montbéliarde breeds. This very broad-ranging programme will make it possible to optimise the choice of breeding animals to be tested. 2003 was the first year when the selection of the majority of calves to be tested was conducted on the basis of the SAM index for 8 production, fertility and disease-resistance traits. To do this, 10,000 typing operations are performed every year to characterise the families and, for candidates, their dams or the selection cores. This programme, managed by INRA and a breeders' cooperative, the UNCEIA, associated with a private company, LABOGENA, is the only one of its type in the world and can only be implemented thanks to the original organisational structure of the French genetic selection system. In addition, it is an exceptional resource for the characterisation of genes and the investigation of interesting mutations.

Individual evaluation
Calves produced as a result of planned mating are bred in individual testing stations, in order to select, on the basis of conformation, growth and food efficiency criteria, those that should be destined for progeny testing.

In the case of hardy breeds, in which testing programmes do not exist, individual evaluation of young bulls intended for artificial insemination (AI) or natural service, is essential. It is for this reason that selection units select the best 3 or 4 young bulls produced by breeding centres with a view to their use in AI; the others are intended for either natural service or are eliminated.

Depending on the breeds, the number of young bulls that have been the subject of effective evaluation of individual performance in accordance with the INRA - Institut de l'Elevage protocol, is variable (table 4). The data indicate that:
- 1503 young bulls have been individually evaluated in stations. This number has varied little over the last few years (1,503 in 2001 and 1,373 in 2002);
- almost all bulls in beef breeds are individually evaluated prior to being tested, since the selection pressure exerted in stations is very high (one bull retained for every three bulls entering the station). Evaluation stations are used for breeds for which there is no progeny testing.

Table 4: Activity of individual testing stations in 2003 (compared to 2002): number of young bulls assessed for their individual performance
(sources: UNCEIA)

Breeds	Young bulls			
	Entered		Retained	
	2003	2002	**2003**	2002
Normande	**388**	383	**154**	157
Montbéliarde	**426**	421	**162**	159
Abondance	**43**	19	**15**	17
Tarentaise	**25**	18	**14**	14
Charolaise	**132**	127	**35**	68
Limousine	**42**	42	**22**	12
Blonde d'Aquitaine	**57**	48	**15**	12
Rouge des Prés (Evaluation station)	**77**	65	**4**	4
Bazadaise *(Evaluation station)*	**6**	0	**2**	0
Aubrac *(centre d'élevage)*	**133**	110	**3**	3
Gasconne *(breeding centre)*	**70**	62	**3**	3
Salers *(breeding centre)*	**90**	61	**2**	1
INRA 95	**14**	17	**8**	0
Dairy breeds [(1)]	**882**	841	**345**	347
Specialised beef breeds [(2)]	**231**	217	**72**	92
Breeding centres [(3)]	**376**	298	**14**	11
Strain [(4)]	**14**	17	**8**	0
OVERALL TOTAL	**1503**	1373	**439**	450

[(1)] *Normande, Montbéliarde, Abondance, Tarentaise*
[(2)] *Charolaise, Limousine, Blonde d'Aquitaine*
[(3)] *Maine-Anjou, Bazadaise, Aubrac, Gasconne, Salers*
[(4)] *Inra 95*

Progeny evaluation
Progeny testing operations are the keystone of selection programmes. Organisation of the selection base is such that all the bulls tested rapidly reach the required level of precision to be able to safely select sires. To this end, selection programmes for the major breeds are the subject of a rigorous progeny testing protocol, which includes comparative analysis of the effects of a proportion of the progeny testing AIs to check, if necessary, the absence of any bias due to a region effect.

- In dairy breeds, 985 bulls were tested in 2003, versus 980 in 2002. This number has been stable overall since 1990. The progeny testing rate, measured by the ratio of the number of bulls tested over the number of first artificial inseminations (FAI) per bull is one per 3,321 (tables 1 and 2).

  Selection is even more stringent when large numbers of animals are tested. Bulls which have undergone all the tests and have obtained a genetic index known with minimal precision are submitted to a Programme Monitoring Committee. This committee submits its proposal to the Ministry of Agriculture as to whether or not these bulls should be accredited for use in artificial insemination. One in 9 bulls submitted to the monitoring committee is accredited but only one in 13 will then be used intensively in dairy breeds. Even though the total number of bulls used is very high (more than 6000), half of the inseminations performed in each of the major breeds use only a few dozen bulls.

- In beef breeds, 22 bulls were tested in 2003 with respect to their maternal qualities. In addition, 19 progeny are currently being monitored for this programme alone, using on-station female progeny.

Furthermore, 15 bulls are also being tested with respect to their meat aptitudes. In addition, 28 of their progeny are being assessed. These do not include the progeny of Charolais bulls (26 progeny in 2003), which are being assessed for the early muscularity programme. Depending on the types of production, this progeny monitoring is conducted either on-farm or on-station.

The scope of these selection programmes is entirely remarkable and unique in developed cattle rearing countries. It is thanks to the sustained efforts of selection units that these programmes have achieved their current level and have enabled a relative development of insemination in dairy herds. Improver bulls used in insemination cover a very wide range of production types, from calves sold early to young cattle, as is demonstrated by beef cattle genetic assessments.

A.2.3 Quality control of selection

The quality of the programmes and of the work carried out by the selection units can be verified thanks to the genetic assessments calculated every year by INRA and the Institut de l'Elevage. To calculate these genetic assessments there are two possibilities:

- the fixed base: this measures the genetic difference between the dairy genetic quality of AIs performed in a given year with that of the females having produced, born between 1978 and 1981 (with first calving from 1 September 1980);
- the mobile phase, which refers to the index used by breeders. Thus, every year, the population, for which the average index is nil by convention, defines the mobile phase. This population groups together animal insemination bulls:
  * progeny tested in France,
  * with a coefficient of determination (CD) >= 70 and born between 1992 and 1995 for the Montbéliarde, Normande and Prim'Holstein breeds,
  * or with a CD >= 50 and born between 1990 and 1995 for the Abondance, Pie Rouge, French Brown, Tarentaise and French Simmental breeds.

Dairy breeds

The genetic improvement results established over 10 years for the 3 main dairy breeds (table 5), demonstrate an improvement in these genetic assessments over time: for the “Milk” criterion alone, the genetic quality of the dairy cows assessed has increased by almost 107 kg/year in Holsteins, 75 kg/year in Normandes and 69 kg/year in Montbéliardes. This increase in genetic quality largely explains the annual increase in the production of cows registered for milk recording since a reduction in the herd effect is being observed for each of these breeds.

Milk recording provides an objective measurement of what the farmer observes. The values observed for the various parameters measured express the resultant between the genetic effect imputable to AI and the effect due to herd behaviour. Most of the progress is explained by genetics rather than by the herd effect, which is weakening. The genetic assessment column indicates the average result by AI in the herd.

Table 5: Genetic improvement assessment over 10 years (1993-2003)
(sources: Institut de l’Elevage)

		Genetic quality	Herd effect	Milk recording	Genetic assessment of FAIs
Prim'Holstein					
Improvement	INEL (points/year)	4,58			5,1
	TP (g/kg/year)	0,07	-0,02	0,08	0,05
	TB (g/kg/year)	-0,11	0,06	-0,02	-0,18
	Milk (kg/year)	106,9	-11,4	108,2	129,4
	Protein (kg/year)	4	-0,4	4	4,5
	Fat (kg/year)	3,3	-0,1	4,2	3,6
Normande					
Improvement	INEL (points/year)	3,86			4
	TP (g/kg/year)	0,1	-0,03	0,1	0,02

		Genetic quality	Herd effect	Milk recording	Genetic assessment of FAIs
	TB (g/kg/year)	-0,01	0	0,01	-0,05
	Milk (kg/year)	74,8	-13,7	54,3	92,2
	Protein (kg/year)	3,2	-0,7	2,4	3,3
	Fat (kg/year)	3,2	-0,6	2,5	3,8
Montbéliarde					
Improvement	INEL (points/year)	3,03			3,2
	TP (g/kg/year)	0,03	-0,01	0,04	-0,01
	TB (g/kg/year)	0	0,04	0,05	-0,1
	Milk (kg/year)	68,8	-13,8	50,4	87,5
	Protein (kg/year)	2,5	-0,6	1,9	2,8
	Fat (kg/year)	2,8	-0,4	2,2	2,8

FAI: first artificial insemination
INEL : "index économique laitier" (French dairy economic index)
TP : protein rate
TB : fat rate (taux butyreux)

Beef breeds

The results for the various types of production (tables 6 and 7) in the three major beef breeds demonstrate the quality of the sires proposed to breeders and the zootechnical choices made by the latter for the reproduction of the products desired. These choices differ according to breeds and also vary over time.

In beef breeds, the indices are centred around 100. The mean value of indices is therefore expressed relative to 100 on the basis of the means and standard deviations of each series, which are compared to controls. The columns for which the gains are the most significant correspond to the main objectives of selection. The maternal qualities represent a synthesis of the criteria from other columns (fertility, calving, lactation).

Charolais

Station
Limousin

Young cattle
Station

Table 6: Meat production, by breed and type of production
(sources: Institut de l'Elevage – INRA)

Breeds	Type of production	Ease of birth		Weight at a standard age		Conformation		Carcass yield		Meat aptitudes		First artificial insemination	
		1993	**2003**	1993	**2003**	1993	**2003**	1993	**2003**	1993	**2003**	1993	**2003**
Charolais	Calving aptitudes	102	**106**			116	**124**					247 570	**202 090**
	Young cattle Station	101	**101**	102	**103**	103	**104**	104	**103**	104	**105**	399 760	**241 560**
Limousin	Calves for slaughter Industrial farming unit	98	**102**	107	**102**	114	**109**	107	**104**	111	**106**	151 410	**140 180**
	Young cattle Station	98	**97**	99	**102**	105	**107**	101	**100**	103	**103**	183 230	**105 390**

Breeds	Type of production	Ease of birth		Weight at a standard age		Conformation		Carcass yield		Meat aptitudes		First artificial insemination	
		1993	**2003**	1993	**2003**	1993	**2003**	1993	**2003**	1993	**2003**	1993	**2003**
Blonde d'Aquitaine	Calves for slaughter Industrial farming unit	100	**103**	106	**103**	108	**106**	104	**103**	106	**106**	130 120	**39 760**
	Young cattle Station	97	**102**	99	**103**	107	**109**	103	**103**	101	**105**	110 340	**94 810**
INRA 95	Calves for slaughter Industrial farming unit	102	**103**	99	**107**	128	**133**	107	**118**	113	**116**	71 240	**57 330**

Table 7 presents the average genetic value of inseminations performed using bulls accredited for the production of renewal females in the three major French beef breeds. Once again, the objectives of selection conducted for each race correspond to the choices of farmers.

In beef breeds, the indices are centred around 100. The mean value of indices is therefore expressed relative to 100 on the basis of the means and standard deviations of each series, which are compared to controls.

Tableau 7: Mean genetic value of inseminations of breeding females
(sources: Institut de l'Elevage – INRA)

Breeds	Growth Development		Fertility		Calving		Lactation		Maternal qualities		First artificial insemination	
	1993	**2003**	1993	**2003**	1993	**2003**	1993	**2003**	1993	**2003**	1993	**2003**
Charolais	101	**106**	101	**105**	107	**106**	105	**108**	106	**110**	172 520	**213 770**
Limousin	98	**115**	103	**104**	104	**100**	103	**112**	103	**116**	64 540	**73 330**
Blonde d'Aquitaine	98	**104**	100	**108**	100	**101**	108	**103**	103	**107**	86 960	**82 630**

Figure 4 presents the historical evolution in milk and meat programmes. The numbers of bulls tested and the associated genetic effort are indicated for dairy breeds and beef breeds. The evolution in this genetic effort is demonstrated by an index calculated such that it increases as the number of FAIs/bull tested falls.

The top curves represent data for dairy breeds. The first curve represents the evolution in the number of dairy bulls tested per year. The fall in the slope since 1991 reflects a reduction in the size of the programmes, which depends on requirements and hence on the inseminations performed. The second curve represents the evolution in the selection effort, which is reflected by the ratio between the number of AIs and the number of bulls tested. It represents the investment capacity of selection units. For example, on average, one bull is tested for 3800 Holstein AIs and one bull for 2500 Normande AIs.

The lower curves reflect the same data but for beef breeds. A reduction in the number of bulls tested is observed (1970-1977 period), followed by a stagnation which has been continuing for many years. The almost nil slope of the curve reflects the constant nature of investments in terms of selection efforts.

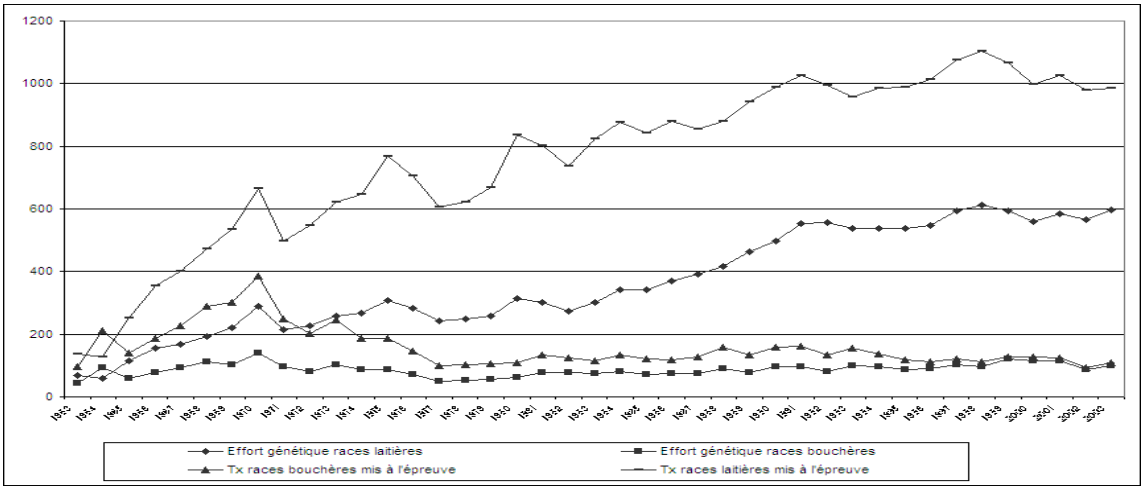

Figure 4: Historical evolution in dairy breed selection programmes (top 2 curves) and beef breed selection programmes (bottom 2 curves) between 1963 and 2003

Genetic effort dairy breeds — Genetic effort beef breeds
Beef breed bulls tested — Dairy breed bulls tested

A.2.4 Calculation of genetic evaluations of breeding animals

Calculation of the genetic evaluation of sires is an eminently scientific operation. To be effective, it requires that a great deal of information be processed using suitable statistical methods with the objective of seeking the best compromise between the specificity of the model and real data. In France, all genetic evaluations are under the direct responsibility of genetic scientists at INRA, which have access to all the data centralised at the national site of INRA's CTIG.

In concrete terms, for all the traits and in all species, the "BLUP animal model", which takes into account numerous non-genetic variation factors is systematically applied.

The official circulation of genetic value indices is conducted by the Institut de l'Elevage for cattle, sheep and goats. This is intended to ensure the objectivity of results and the independence of breeding animal owners or of the structures directly concerned by genetic or commercial use of the results.

Experts from national technical institutes have been very involved in the work since the start, and in bodies having for several years enabled international genetic evaluation of dairy bulls in the context of Interbull (a sub-committee of ICAR: International Committee for Animal Recording), entrusted with the task of harmonising the genetic evaluation of cattle between various countries and performing international classification of animals.

INRA and the national technical institutes are recognised as official bodies by the European Union, authorised to conduct and circulate the results of genetic evaluations. France has been involved in the international classification systems set up by Interbull for the Holstein, French Brown and Simmental breeds since 1995.

A.2.5 Specific features of the system to organise selection of beef cattle breeds in France

The organisational structure for the creation and dissemination of genetic improvement in the case of beef cattle breeds is based on two approaches:

- firstly, selection and large-scale dissemination by AI of semen from carefully evaluated bulls selected from those tested in selection programmes (breeding and insemination);
- secondly, assistance for breeders to help them identify natural service bulls liable to improve the production of dairy herds.

In this respect, monitoring of beef performance plays a very important role, since 15% of French milking cows are controlled (635,000) in 12,000 herds. This selection base including identified animals,

with a known civil status, subject to performance monitoring (weighing and scoring on weaning) is used:

- firstly for collective programmes, with a view to recruiting future breeding animals (bull dams and future bulls to be tested) and evaluating the progeny of the breeding animals tested, particularly in terms of ease of birth and pre-weaning performance;
- secondly, to evaluate on the basis of on-farm results all those breeding results for which performance monitoring information is available in order to genetically manage herds and direct dissemination through natural service.

This last part is entirely original since, in order to obtain on-farm genetic evaluations of their breeding animals (IBOVAL), breeders must connect their herds by having a certain percentage of insemination performed so that the performance recorded in the herds is comparable at a breed level. Under these conditions, indices are published on the main aptitudes (ease of birth, growth before weaning, muscle and bone development, ease of calving, maternal aptitude, lactation) for the cows, bulls and calves of each herd. National tools for gathering this information have been developed, i.e. the genetic assessment of a dairy herd, individual performance sheets for males and females, along with index lists.

In addition, an on-station growth performance evaluation system has been organised via breed societies (UPRA) consisting in evaluating the muscle growth, feed capacity and morphology of bulls intended primarily for natural service but which may also be recruited into collective programmes. These animals must follow a protocol (recruitment conditions on entry, growth regime, etc.) and a genetic evaluation of their intra-station performance must be available.

All the genetic evaluations (bulls tested and indexed in the context of collective schemes, animals indexed on-farm by IBOVAL, animals evaluated on-station) are the subject of a **breed qualification** which aids the better use of breeding animals in the collective scheme and in dissemination by natural service.

Regular monitoring of the genetic efficacy of the system is organised via the calculation of zootechnical assessments (genetic values of each insemination) along with the observation of various parameters concerning zootechnical performance (growth, birth weight, morphology, etc.).

A.3 REPRODUCTIVE TECHNIQUES FOR THE GENETIC IMPROVEMENT OF LIVESTOCK BREEDS

In species of zootechnical interest, genetic improvement depends on the effectiveness of reproductive techniques just as much as it does on the precision with which the phenotypic traits of individuals can be evaluated.

Artificial insemination

Artificial insemination was the first technique used on a large scale to accelerate the dissemination of better genetic material. This technique is exploited to various degrees in the majority of species of livestock.

In cattle, 4,372,171 FAIs (first inseminations) were performed in 2003, including 3,291,918 in dairy breeds and 1,080,253 in beef breeds (table 8).

Table 8: Breakdown of animal insemination according to species
(data: UNCEIA [(1)], ANIO [(2)], CAPRI-IA [(3)] and ITP [(4)])

	CATTLE (1)		SHEEP (2)	GOATS (3)	PIGS (4)
	Dairy breeds	Beef breeds			
Year 2003	3 291 918	1 080 253	870 956	71 343	5 794 812

Embryo transfer

Embryo transfer is another technique widely used in livestock breeding. These embryos may be produced by in vivo fertilisation in super-ovulated animals or be obtained in the laboratory following collection of oocytes and in vitro fertilisation.

The statistics for 2003 show that 5,665 donor cows were used and that 33,729 embryos, including 99% produced in vivo were transferred in France (table 9).

Table 9: Embryo transfer activity in France (source: AETE)

Embryo production	***2003***	***var / 2002***	***2002***
In vivo			
Number of donors	5 665	-16,7%	6 797
Number of embryos collected	64 925	-1,7%	66 031
Number of transferable embryos	**37 433**	**-0,8%**	**37 725**
Number of embryos collected per donor	11,46	18,0%	9,71
Number of transferable embryos per donor	6,61	19,1%	5,55
% transferable embryos/embryos collected	58%	0,9%	57%
***In vitro* (OPU)**			
Number of oocyte donors	77	-9,4%	85
Number of OPU sessions	77	-46,5%	144
Number of transferable embryos	**261**	**-12,4%**	**298**
Number of embryos transferable per session	3,39	63,8%	2,07
Total in vitro embryos	***261***	**-12,4%**	***298***
Total number of transferable embryos	**37 694**	**-0,9%**	**38 023**
Embryo transfer			
In vivo			
Fresh	18 415	4,9%	17 562
Frozen	15 076	-0,5%	15 158
In vitro			
Fresh	231	9,0%	212
Frozen	7	-22,2%	9
Total number of embryos transferred	**33 729**	**2,4%**	**32 941**
% *in vitro* embryos transferred	0,7%	7,7%	0,7%
% frozen embryos transferred	44,7%	-0,6%	46,0%

For conventional transfer (embryos produced in vivo), the development of more effective superovulation protocols has led to significant improvements in the overall yield, which is currently 6.7 transferable embryos per collection.

Embryos can also be obtained by the collection of oocytes from ovaries in abattoirs or from live animals by harvesting from the ovaries with ultrasound control (OPU technique: ovum pick up). The oocytes harvested in this way are subjected to successive maturation, fertilisation and culturing stages in vitro, which lead to embryos in the blastocyst stage being obtained, which can then be transferred into recipient females. A genetically interesting donor cow can thus theoretically produce up to 200 calves using surrogate mothers.

Although the embryo yields obtained under these conditions (4 to 5 embryos per collection) have significantly improved in recent years, the cost of producing these embryos in vitro is still around three times higher than the cost of embryos produced in vivo. Since freezing of these embryos has still not been mastered, the use of in vitro embryos remains linked to research programmes or very limited applications in the field *(*Montbéliarde breed).

Cloning

Reproduction by cloning is a logical addition to the techniques used currently. The potential positive impact of cloning on genetic improvement justifies careful examination of the conditions for implementation of this technique.

The most logical approach is cloning of adult animals, for which the genetic performance is known and validated.

However, the value of cloning appears to be relatively limited for dairy breeds. In fact, a bull derived from the clone of an indexed bull would, right from its birth, have a genetic progress handicap corresponding to what is conventionally termed “the progression of progress per year”, i.e. 110 kg of milk per year for the Prim’Holstein breed for example. Insofar as there will necessarily be 3 years between the time when a cell is harvested from the animal to be cloned and the time when semen from the clone will become available, the cloned animal will be outdistanced, by 350 to 400 kg of milk, by bulls having performed a generation of n+3 in comparison to it.

For this reason, it is therefore evident that, for the time being, cloning techniques remain limited, at least in theory, to hypotheses for their use in programmes in which there is little genetic progress, such as for beef breeds, for example, or for industrial crossbreeding bulls.

Otherwise, it may be possible to envisage cloning embryos produced from parents with interesting genetic traits. This approach has not yet found any practical application due to the excessively low yield of cloning but also because the genetic properties of the embryos are not really known. There could be a resurgence of interest in this method if selection based on examination of a large number of relevant genetic markers could be performed on embryos.

Genetic selection of embryos on the basis of markers, independent of cloning, is now a reality. This involves determination of the sex and the presence of a few alleles of genes with important and known effects. The analysis of a large number of markers requires the use of a relatively high number of embryo cells. Cloning makes it possible to massively multiply the few cells that can be harvested from an embryo without any risk of compromising the embryo’s survival.

It is also conceivable that cloning could help accelerate the dissemination of the genomes of selected breeding animals via reproduction of their direct progeny by cloning.

GLOSSARY

Alleles
Different versions of the same gene. The various alleles direct synthesis of proteins with structures and biological activities that differ to various extents. The distribution of alleles defines the traits of an individual.

Aneuploid
Said of a cell with an abnormal number of chromosomes.

Blastocyst
Embryo composed of 64 or 128 cells.

Chimera
Living organism formed by cells from several organisms, of the same species or otherwise. For example: a chimera is obtained following transfer of cells from one embryo into another embryo.

Chromosome
Combination of DNA and proteins present in the nucleus of cells.

Clone
Living organism obtained without sexual reproduction and containing the same genetic material as its parent.

Cloning (in the sense in which it is used in this study)
Operation consisting in enabling the birth of a living organism without sexual reproduction. In animals, cloning is possible by transferring the nucleus of a differentiated cell into an enucleated oocyte.

Cytoplasm
Inner part of the cell surrounding the nucleus.

Differentiation
Natural process transforming totipotent cells into pluripotent, multipotent then completely specialised cells. Differentiation is largely irreversible. Cloning by nucleus transfer enables artificial dedifferentiation of the cells, which return to a totipotent state.

Diploid
Said of a cell that possesses two sets of chromosomes (somatic cells making up the organs).

Embryonic stem cells
Line of pluripotent cells (ES cells).

Enucleation
Operation that consists in mechanically removing the nucleus from an oocyte (more generally from a cell).

Epigenesis
Set of mechanisms not involving modification of the genetic message and which control the capacity of a gene to be activated or otherwise by its natural inducers. For example: the methylation of certain DNA bases reversibly inactivates a gene.

Epimutation
Hereditary modification of genes, which alters their expression but not their primary structure.

Evolution
Processes based on spontaneous mutations of genes and natural selection having led to the emergence of new species.

Gametes
Sex cells, germinal cells: oocytes and spermatozoa.

Gene
Coded message supported on DNA, itself present in the chromosomes. Decoding of a gene gives rise to a protein.

Genetic code
Code indicating the correspondence between the succession of bases of a gene and the order of the amino acids in the corresponding protein.

Genetic engineering
Set of operations enabling the manipulation of genes: isolation, mutation, gene construction, gene transfer to cells or whole organisms.

Genetic selection
Operation consisting in only retaining the individuals of a species which best meet the requirements of the scientist.

Genome
Set of genes in a living organism.

Genotypic
Said of the effects of heredity on a living organism.

GMO
Organism that has been genetically modified by the intervention of a scientist.

Haploid
Said of a cell that possesses only one set of chromosomes (sex cells).

Hybrid
Living organism obtained by crossing two organisms belonging to different varieties, breeds or species. For example: maize hybrids, the mule.

Laws of heredity
Set of laws defining the mode of transmission of genetic traits from one generation to the next.

LOS
Large offspring syndrome. Set of abnormalities characterising newborn cloned animals.

Mitochondrion
Mitochondria are small structures (organelles) located in the cytoplasm of most animal and plant cells. They provide the energy required to make the cell function. The number of mitochondria in a cell (several units to several tens of thousands) depends on the intensity of its activity: a muscle cell, for example, possesses a large number.

Morula
Embryo composed of 16 or 32 cells.

Multipotent cell
Cell capable of participating in the formation of a restricted number of organs. For example: bone marrow stem cells which give rise to red and white blood cells.

Mutant
Organism in which certain alleles have modified the biological properties.

Natural selection
Process that only retains those individuals and species best suited to the conditions of their environment.

Nuclear reprogramming
Set of phenomena enabling the genes of a cell to acquire a new sensitivity to cell inducers.

Oocyte
Female sex cell, also called an egg cell.

Organ stem cells
Multipotent cell lines.

Parent (in the sense in which it is used in this study)
Individual donating a nucleus for the generation of a clone.

Penetrance
Capacity of a gene carrying a well defined trait to express itself phenotypically in a population.

Phenotypic
Said of the combined effects of heredity and environment on a living organism.

Pluripotent cell
Cell capable of participating in the formation of any type of organ in a living organism but incapable of giving rise to a living organism on its own.

Protein
Macromolecule formed of amino acids linked in a specific order. This order is defined by the order of the bases of the corresponding gene. The order of amino acids also determines the form and activity of the protein.

QTL:"QUANTITATIVE TRAIT LOCI"
Locus of a genome containing genes responsible for a hereditary trait with a marked phenotypic impact in livestock.

Sequencing
Operation consisting in determining the order of the amino acids in a protein or the order of the bases of a gene and, more generally, a DNA fragment.

Sex cell
Gamete, germinal cells.

Somatic cell
Non-sexual cell, organ cell.

Telomeres
Short sequences of DNA found at the extremities of chromosomes. Telomeres protect DNA and prevent digestion of its extremities. Aging cells have shorter and shorter telomeres providing less and less protection to the DNA.

Totipotent cell
Cell capable of giving rise to a living organism.

Transgenesis
Transfer of a gene to a living organism, leading to a line being obtained that carries exogenous genetic information, which may come from the same species or a different species

Unipotent cell

Cell that is only capable of participating in the formation of a single organ. For example: the precursor cells of spermatozoa.

Zygote

Fertilised oocyte.

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