The Production and Potential Use of Transgenic Livestock

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Recent technological advancements in biology are expected to contribute most importantly to the cattle artificial breeding and embryo transfer industries and ultimately to meat and milk production. At present, nearly 70% of 11 million U.S. dairy cows are mated by artificial insemination. Genetic improvement in dairy cattle through artificial insemination has resulted in a doubling of milk production per cow over the past 30 years. This tool for genetic improvement has received little use in meat animal production. While technically feasible, efficient systems for use of AI on range animals have not been applied. Little progress has been made in genetic improvement by selection of offspring from superior females because a cow produces relatively few calves per year or in a lifetime. To multiply the progeny from an outstanding female or mating, a commercial embryo transfer industry has developed. This industry in 1984 performed approximately 100,000 transfers in the USA and 200,000 in the world, of which approximately 25% were with frozen embryos. Its present armament of technologies includes the ability to superovulate cows, collect and transfer embryos nonsurgically, store embryos frozen and divide each embryo into two viable embryos. At present 20 to 30 calves can be produced from one valuable donor cow/year by use of embryo transfer. This industry provides the delivery vehicle in domestic animals for newly emerging biotechnologies, such as embryo cloning, sexing and gene transfer.

Gene Transfer

Strong interest in producing transgenic livestock occurred following the first production of transgenic mice by Gordon and Ruddle (1981). This interest was greatly enhanced by the experiments of Palmiter and Brinster and colleagues, demonstrating that insertion of a linear fragment of a plasmid containing a metallothionein-I promoter and rat growth hormone (Palmiter et al., 1982) or human growth hormone (Palmiter et al., 1983) into the genome of murine embryos perturbed growth regulatory mechanisms sufficiently to cause as much as a two-fold increase in growth.

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While most productivity traits are influenced by several genes, these experiments demonstrated that a gene coding for a product which perturbs a hemeostatically maintained regulatory system or a gene coding for a rate limiting step in a process can cause significant change in the production of a product.

Several genes are of interest for insertion into the genome of livestock. These include growth hormone with its potential for increasing growth and lactation. In dairy cattle, injection of growth hormone has resulted in an increase of approximately 15% to 40% in milk production (Bines et al., 1980; Peel et al, 1981, 1985; Gorewit et al., 1982; Bauman et al., 1985). The giant mouse gene identified by Bradford (1982) might also produce enhanced growth in livestock. Specific genes affecting components of reproduction have been identified. For example, the Booroola gene, when present, causes the Australian merino sheep to increase ovulation rate and to produce multiple births rather than single lambs (Davis et al., 1982). This gene is presently being cloned in Australia as well as two genes controlling wool growth. In mice, Spearow (1984) has identified genes influencing ovulation rate which increase ovulation up to 6-fold. If the Booroola gene were inserted into cattle or the Spearow gene into swine, it might be possible to reliably produce twins in cattle or increase litter size in swine. It may also be possible to impart resistance to specific diseases of animals or birds through transfer of genes. Resistance to some diseases of humans and mice (Bach, 1982) or Mareks disease in poultry (Payne, 1973) are known to be controlled by single gene loci. Cloned genes of the major histocompatibility complex and genes for interferon offer hope for enhancing immune protection from disease. One gene of the MHC complex, SLA-PD1, has been cloned (Singer et al., 1982) and shown to be expressed in transgenic mice (Frels et al., 1985). The MHC gene (SLA-PD1) has been introduced into the genome of swine, sheep and rabbits at the USDA Beltsville laboratories but its effect on immune response is yet untested.

Genes Promoting Growth

The genes being studied at the present as additions to the genome of domestic animals for the purpose of enhancing growth or lactation are the genes coding for protein hormones known to be involved in normal growth regulation.

In animals, growth is controlled by a complex interaction of polypeptide hormones produced in the hypothalamus, pituitary gland and liver. Growth hormone (GH), an intermediate in this array, is produced in the somatotrophs of the

pituitary gland and is under the neurohumoral regulation of two hypothalamic peptides, somatostatin (SS) and growth hormone releasing factor (GRF). Somatostatin inhibits the release of GH while GRF stimulates both GH synthesis and release. GH is not thought to mediate growth directly. It acts by stimulating the synthesis and secretion of insulin-like growth factor-1 (IGF-1), which then mediates postnatal somatic growth by binding to membrane receptors of peripheral mesenchymal cells.

Genes coding for these peptides have been isolated and cloned and large quantities of highly purified preparations are available. Administration of foreign GH and GRF to a number of species enhances some growth characteristics; however, the necessity of continual administration of such preparations of livestock would be inconvenient and costly. With the advent of gene transfer techniques by microinjection of eggs (Brinster et al., 1985), the possibility of creating animals containing these genes has been realized.

The growth response occurring from animals made transgenic for one of the growth regulatory genes or administered the growth promoting gene product appears to differ among species. For example, injected growth hormone produces large increases in the growth rate of rodents and as much as a 163% increase in fish (Higgs et al., 1975) but only modest growth increases in swine (15%, Machlin, 1972; 10%, Chung et al., 1985) and sheep (20%, Wagner and Veenhuzen, 1978, 0% Muir et al., 1985) or no growth increase in chickens (Scanes et al., 1984; Burke and Builder, 1985). Conversely, the same growth hormone injected into lactating dairy cows has increased lactation 15% to 40% (Bauman et al, 1985).

Growth response from animals transgenic for these same growth hormones is not likely to be different from the response achieved by frequent injections of the growth hormone. While mice transgenic for rat, human or bovine growth hormone have grown much faster and larger than normal (Hammer et al., 1985b), increased growth rate did not occur for pigs transgenic for the human growth hormone (Hammer et al., 1985a). Genes for other hormones in the growth hormone regulatory sequence, such as growth hormone releasing factor (GRF) or insulin-like growth factor 1 (IGF_1), might provide a more effective growth signal. The production of mice transgenic for a human metallothionein-growth hormone releasing factor (mthGRF) resulted in greatly increased growth but this was accompanied by overstimulation of pituitary somatotrophins and resulted in pituitary tumors (Hammer et al., 1985b). As yet, the growth response to insulin-like growth factor (IGF_1 or somatomedin C) has not been tested by injection of the hormone or by production of animals transgenic for the IGF_1 gene, although the genes for IGF_1 and IGF_{II} have been cloned.

In domestic animals, birds and fish, growth is also controlled in part by chromosomes other than the growth hormones (Scanes, 1985). These include the thyroid hormone in birds (Scanes, 1985) and gonadal steroids in fish (Malison et al., 1986).

Development of Methods for Gene Transfer in Domestic Animals

The DNA or gene which is microinjected into the pronuclei of mammalian zygotes is usually in large copy number (100-1,000) and prepared by enzymatically cutting into a linear fragment, a circular plasmid containing genomic sequences of the intended gene, an inserted gene promoter and a small portion of the bacteria in which the gene was cloned. In mice, this linear gene fragment would be microinjected usually but not necessarily into the male pronucleus of a zygote resulting from recently-fertilized eggs. The zygote is then transferred to the oviduct of a recipient mouse or cultured in vitro to a late-stage embryo and transferred into the uterus. Nuclei of late-stage embryos can be microinjected but the offspring are chimeric in expression of the gene. The efficiency of gene transfer by microinjection into pronuclei is low. Integration of the exogenous gene into the genome of offspring occurs for approximately 25% of the embryos microinjected and approximately half of these express the integrated gene. Alone, this should mean that 12% to 15% of the offspring born express the transgenic gene. Unfortunately, the intervening reproductive steps and losses from microinjection reduce the efficiency to the extent that only 1% to 2% of the zygotes receiving gene transfer will result in offspring integrating and expressing the transgenic gene. These efficiencies can be even lower for domestic animals.

Gene transfer in domestic animals has been slower in development relative to mice for technological reasons; such as problems in visualizing the pronuclei into which genes are injected, and development of pronuclear embryos to a stage that can be transferred nonsurgically to recipient cows. As previously discussed, these problems have been overcome and gene transfer can now be successfully performed in cattle. The only report thus far of a gene expressed after transfer into bovine embryos is the transfer of the thymidine kinase gene and its assay in bovine embryos. Approximately 30% of bovine embryos derived from in-vitro or in-vivo fertilizations express the gene after transfer into a pronucleus (Lohse et al., 1985).

Calves have been produced from embryos after microinjection of a gene into the pronuclei of zygotes from which the embryos developed but thus far none have expressed the gene and are thus not transgenic offspring (Kraemer et al., 1985).

Through the use of sheep oviducts to incubate embryos from the pronuclear stage of gene microinjection to transferable morula or blastocyst stage, cattle and sheep transgenic for growth hormone are presently being produced in at least three laboratories.

Offspring expressing an exogenous growth hormone gene have been produced in swine at USDA, Beltsville. These offspring exhibit increased levels of the exogenous growth hormone in their blood but thus far have not grown faster and are not larger than controls (Hammer et al., 1985b). This fact suggests the need for research to understand the regulation of growth in swine.

The production of transgenic livestock is of low efficiency. Based on experiments with mice and depending on the efficiencies of reproductive steps in each species, it is estimated that a gene must be injected into 50 to 200 pronuclei to produce one offspring which has integrated and expressed the gene. Additionally, experiments in mice indicate that one must generate individuals homozygous for the foreign gene to screen for insertional mutations. These are usually lethal

and are caused by the disruption of a native gene. The frequency of insertional mutagenesis in mice is approximately 1 out of 7 to 1 out of 15 (Jenkins et al., 1981; Lacy et al., 1983; Jaenish, 1980; Schnieke et al., 1983; Wagner et al., 1983; Palmiter et al., 1984).

More efficient methods for gene transfer and methods for transfer at a stage more compatible with commercial bovine embryo transfer are being sought. These explorations include the potential use of replication defective retroviral vectors to infect genes into late stage embryos (Mann et al., 1983; Emerman and Temin, 1984a, b; Bandyopaddhyay and Temin, 1984a, b; Sorge et al., 1985).

Retroviral Vectors

Production of transgenic livestock involves the insertion of an exogenous gene into the genome of the production animal. There are potentially a number of methods to introduce genes into embryos; however, currently the majority of gene transfers are peformed with one of two methods - i.e., gene injection or retroviral infection (Palmiter and Brinster, 1985; Brinster et al., 1985). Of these two methods, gene injection of 1 to 2-cell embryos is the major method being used to produce transgenic animals.

The gene injection technique which is the most commonly used method to produce transgenic mice is technologically easy and obviously it works. The technique involves growing the plasmid containing the exogenous gene in bacterial culture (overnight), extracting and purifying the plasmic (3 days) and injecting the linearized plasmid into the pronuclei of 1-cell embryos (Gordon and Ruddle, 1981; Costantini and Lacy, 1981; Wagner et al., 1981; Brinster et al., 1985). However, the gene injection method also has some disadvantages which may be important to analyze with regard to producing transgenic livestock: (1) It is inefficient with 0.1% to 1.0% of injected embryos producing viable transgenic offspring. This obviously is not a large problem if a large supply of inexpensively obtained embryos is available, such as with mice. (2) The method is micromanipulatively tedious, involving individual treatments of each embryo. (3) The injection procedure is invasive and therefore potentially destructive. (4) Early embryos (1-2 cell) are required, which are not easily obtained from livestock species. (5) Genetic disruption (mutations, cancers, lethality) commonly occurs (Wagner et al., 1983; Woychik et al., 1985). (6) There is no control over the number of genes inserted per genome and multiple copies of the gene may be inserted in tandem arrays.

Due to the problems described above with both the gene injection technique and the lower numbers of viable embryos available in livestock species, our laboratory in collaboration with personnel in the retroviral laboratory of Dr. H.M. Temin has been investigating the potential of retroviral vectors to produce transgenic livestock.

Retroviruses are diploid RNA viruses. After infecting a cell, the viral RNA is converted into DNA by a viral enzyme, reverse transcriptase, present in the coat of the virus. The viral DNA circularizes and integrates into the genome of the host cell. The viral genes are transcribed and translated and the viral genome transcribed and packaged as RNA in the protein coat. The rationale for using retroviruses as vectors is obvious. If retroviruses insert their own genes into a host cell genome, then they will also insert a foreign gene that has been placed in the viral genome. This indeed occurs and retroviruses have now been used on many occasions to insert exogenous genes into cells in culture - e.g., hematopoietic stem cells (Eglitis et al, 1985; Hock and Miller, 1986) and zona pellucida free early stage (morula) embryos (Jähner et al., 1985; Huszar et al., 1985; van der Putten et al., 1985). The fact that the vector is infectious has also allowed the production of transgenic mice by infecting midgestation embryos and blastocysts (Jaenisch et al., 1981; Stuhlmann et al., 1984).

The use of retroviruses may therefore solve some of the problems inherent in the gene injection technique, especially with regard to producing transgenic livestock. Some of these advantages may be: (1) Retroviral infection of embryos is more efficient in that 2% to 50% of infected embryos (depending on the stage and treatment of embryos) produce viable transgenic animals (mice). It is thus possible to obtain transgenic animals using fewer embryos, which is an important consideration in livestock research. (2) 1-cell embryos are not needed. (3) Genetic disruption of the embryos is a low probability. (4) Only single copies of the provirus, and therefore the exogenous gene, are inserted per genome (although different sites of insertion are possible and at present there is no control over the site of insertion).

There are, of course, some problems and disadvantages of using infectious retroviruses as gene vectors: (1) If 1-cell embryos are not used, the potential is high to produce transgenic mosaic animals, i.e., the exogenous gene is not in every cell or every tissue of the animal. This may or may not be a problem, depending on the expression of the gene and the planned use for the transgenic animals. (2) Many retroviruses are pathogenic and cause cancer and it is not desirable to use potentially harmful organisms to produce transgenic food-producing animals. To circumvent this problem, replication defective helper free retroviral vector systems have been developed. These will be explained shortly. (3) Retroviral genetics and construction of vectors is technologically more complex than plasmid constructs and introduces another level of expertise required in developing transgenic animals, and finally (4) Retroviruses have cellular and species specificity. It may be necessary to develop more than one vector type to infect different livestock species.

Thus both systems (i.e., gene injection and retroviral infection) have their advantages and disadvantages. The two systems can be summarized as follows. Gene injection is technically easy but tedious, involving manipulations of each individual embryo; is invasive; is inefficient, requiring large numbers of embryos; and results in developmental problems in injected embryos. Using retroviral vectors is technologically more complex (much more research is yet to be done on producing efficient vectors for different species), but it is not manipulatively tedious; does not involve individual treatments of embryos; is not invasive; is more efficient, therefore requiring fewer embryos; and does not produce as many developmental defects as gene injection techniques.

In order to produce safe retroviral vectors, helper free replication defective vectors have been developed. For a complete explanation of retroviral genetics and the replication defective helper free vectors being developed, the reader is directed to the literature (Mann et al., 1983; Miller et al.,

1985; van der Putten et al., 1985; Wolff and Ruscetti, 1985; Rubenstein et al., 1986; Temin, 1986).

To produce a replication defective retrovirus, the trans acting gene sequences involved in reproducing and packaging the virus (gag, pol, env) are removed and replaced by an exogenous gene of choice. The retroviral LTR's are not altered and the exogenous gene is expressed either from the LTR promoter or an introduced promoter. A DNA copy of this vector is subcloned into a plasmid and transfected into a suitable cell line. The vector can then be rescued by superinfecting the cell line with a helper retrovirus that supplies the packaging signals. The harvested virus is helper present (i.e., both the helper virus plus the replication defective virus are packaged). To produce a helper free system (Mann et al., 1983; Miller et al., 1985; van der Putten et al., 1985; Rubenstein et al., 1986), the trans acting packaging sequences of the retroviral genome are transfected into a cell line (now called a helper cell). Subsequent transfection with the replication defective DNA will result in replication and packaging of only the vector. The virus is harvested (free of helper virus) and, because it is replication defective, can be used as a dead-end vector for inserting genes into cells or embryos.

A final step in producing safe retroviral vectors involves alteration of the LTR of the vector so that the vector DNA is transcriptionally inactive (self inactivating vector) (Yu et al., 1986). This reduces the possible activation of cellular oncogenes and potential rescue by an indigenous retrovirus.

At the present stage of research on the production of transgenic livestock, it cannot be predicted which will be an optimum system to insert genes into the animals (i.e., either or both the gene injection or retroviral techniques may be useful). It is obvious that research will need to progress in both directions. The previous discussion on retroviral vectors has been aimed to introduce the reader to the technology of retrovirus genetics and to present some potential areas where the use of retroviruses may be advantageous in producing transgenic cattle, sheep or pigs.

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Discussion

D. Devol: Could you comment on whether or not transgenic animals can reproduce? Are the traits, are the genes, then passed on to the offspring and is there an advantage in using gene injection versus using retroviruses as far as reproductive traits?

K. Squire: Right! This has all been done in mice because we only have a few transgenic animals other than mice on the ground in Beltsville, but "yes" in mice. They've produced many mice that are transgenic. The gene is in the stem cells of the mice and it is being passed. Now, this does not occur unfailingly in mice. Obviously, the gene has to be in the stem cells. But if it is in the stem cells, the gene can be passed down through many generations. And this has been done with both gene injection and with retroviral insertion as well. Again, one of the reasons that you need a one-cell embryo is because once you get a gene integrated into the one-cell embryo, when that cell splits into cells 2, 4, 8 and so on, every cell of the animal will then have the exogenous gene in it. That is why people are using the one-cell embryo. What I hope will be possible in the future is to put genes into later-stage embryos or the blastosyst, or even neonates. We may also be able to target the stem cells into which the genes are inserted or in which they will be expressed as well.

J. Turner: The question I have concerns the actual way the retrovirus goes into the genome. In particular, what about insertional inactivation of existing genes? When you go in, you may be affecting functional genes. And, also, are you concerned about the very powerful promoters that are used?

Squire: In relation to the first question, yes. Until we can actually zero the gene into an area of an embryo or cell where we know it is not going to cause any disruption, we know there is the potential that the inserted gene may insert into a gene that is necessary for growth and development and thereby destroy it. So, at this particular point in time, we

are going to have that problem whether we use gene injection or retroviral vectors. It seems by using retroviral vectors that we have less of a problem than by using gene injection. And your second question regarding the promoters, we can insert our own promoters so that we can actually block the promoters of the retrovirus itself so that we can override them with our own promoters. Obviously, I think the common theory of a transgenic animal as just involving the gene is not adequate. You need to think about the promoter as well; it's not just the gene, it's the promoter as well.

D.Campion: Kevin, I have one question for you. You indicated that it took from 1981 to 1986 to really get into transgenics from a large animal standpoint and much of what you said in terms of the use of this technology you've prefaced by saying "in the future," or "this is what we are working toward." When might we likely see some of this technology at the production level from whatever source?

Squire: My comment, from about 1981 to now, was actually mean to convey it was very soon after 1981-1982 that laboratories planned on making transgenic livestock but it is taking so long because most of the work is being done using the gene injection technique. In other words, we assumed the gene infection in mouse worked; therefore, it will work in cows, pigs and sheep, and I believe that one of the reasons it has taken so long for us to get transgenic livestock is because we are using gene injection. As for the future and when, I don't know. Beltsville already has transgenic animals on the ground but they are not what they want. In other words, it is not just the technique of getting the gene into the embryo or into the animals, we need to get it into a site where we know it will work and where we know it will not do any damage and where we will be able to control it. And this is going to take many, many years. In the work that we are now doing in Wisconsin, for example, we have already produced transgenic mice using these retrovirus vectors. I've already used the retrovirus vectors on pig embryos and I have them back in my laboratory to test when I return. I think actually making a transgenic animal – livestock – pigs, cow, sheep – it's not going to be too long. We are going to start seeing papers on that very soon but to get the animal to do what we want to do, I think, is going to take quite a few years of research.

Question: How many labs are actually working with large animals – particularly meat animals – the same type of thing that you are thinking about?

Squire: There is the Beltsville laboratory, our laboratory in Madison, Dr. Kramer's laboratory at Texas A&M and another group at the University of California at Davis. They were being funded by GenenTec, I understand, and I'm not quite sure with the change GenenTec had in their funding, if that work is still going on at the University of California. Apart from the Beltsville study, which I believe was published in *Science*, there have been no other publications through refereed journals. But the Texas A&M group has reported the birth of calves that were gene-injected. But they are not transgenic, in other words, the gene was not carried into the embryos; but they have an unrefereed report from a conference proceedings that the calves did survive the gene injection procedure and went through and were developed and born. And there is also a report from Neil First's lab on injecting the thymidine kinase gene into bovine embryos, but the embryos were not then transferred to recipient cows. They were kept in the laboratory for about four days, I believe, and an assay was done to determine whether the embryos had an increase in thymidine kinase levels. They did have an increased level but the laboratory does not know whether those genes were carried into the genome of the embryo or not. In other words, the laboratory does not know whether it actually made transgenic animals or not. So they are the four – the four that I know of.

Question: And none in other countries?

Squire: There has been nothing in the literature. I do know other countries, for example Ireland, Australia and Germany, have funded research proposals and I'm sure they are working on the technique.

Question: Let me follow up the reproduction question just a minute. Am I right? I've heard that the original strain of super mouse has completely died out, that they have not continued to reproduce.

Squire: The original strain – that might be true.

Comment: You've led us to believe that you have strains in your own lab that are reproducing now and you are successful with them.

Question: Which of those four labs have strains that are reproducing successfully?

Squire: These four labs I mentioned are doing research on livestock – transgenic livestock. There are dozens of laboratories in the United States that are working on transgenic mice and for different reasons than all the people in this room are interested in making transgenic animals for. And, to my knowledge, there are many strains of mice that are reproducing and passing the gene on.

Question: So you think the reproduction thing has been solved. Have the problems been solved as far as mice are concerned?

Squire: No, because it will depend again on where the gene is inserted. If it does insert in a place that is going to adversely affect the reproduction of the mouse, or the gene is not in the stem cells, then the gene will not be passed on; and at the moment, we do not have the methods to control the site of insertion of the genes. It's hypothetically possible in the future that we will and there is already major work being done by the genetics laboratory at the University of Wisconsin.