GENE TRANSFER IN CATTLE AND SHEEP: A SUMMARY PERSPECTIVE

H. Niemann1, R. Halter2 and D. Paul2

1Institut für Tierzucht und Tierverhalten (FAL), Mariensee, 31535 Neustadt, Germany
2Fraunhofer Institut für Toxikologie und Molekularbiologie, Nikolai-Fuchs-Str. 2, 30625 Hannover, Germany

SUMMARY

The transfer of foreign DNA via microinjection into pronuclei of bovine and ovine zygotes has resulted in the generation of transgenic progeny. However, the procedure is inefficient and only 0.2% to 2% (in some cases up to 5%) of the injected zygotes develop into transgenic animals. The main purpose of gene transfer in these species has been the alteration of growth performance, modification of lactation, the production of recombinant pharmaceutical proteins for human use in the mammary gland of transgenic animals and to confer resistance to specific diseases. Other perspectives appear at the horizon such as alteration of wool production, the creation of insect resistant sheep to avoid losses of skin and fleece, or the alteration of ruminant microorganisms with the goal to increase efficiency of roughage digestion. The primary goal should be the increase of the efficiency of the gene transfer procedure. Recently, significant progress has been reported in the area of embryonic stem cell (ES) and primordial germ cell (PGC) lines which should permit a highly specific and efficient integration of foreign DNA into the animals' genome.

INTRODUCTION

Gene transfer into the mouse germ line has become a routine procedure, and resulting transgenic mice transmit their transgenes to the offspring and thereby allow a large number of transgenic animals to be produced by regular breeding within a short period of time (Palmiter and Brinster, 1986). In contrast, the generation of transgenic livestock presents particular technical and scientific challenges as the transferred genes should confer distinct economic advantages to the animal breeder. The majority of the gene transfer studies in livestock has been carried out in the pig with various goals (for review see Niemann and Reichelt, 1993). This paper summarizes the results of gene transfer experiments in the two major ruminant species cattle and sheep, in which several investigators have tried to modify the genome to enhance growth performance or to alter lactation mainly in attempts to produce recombinant pharmaceutical proteins for human use in the mammary gland of transgenic animals.

GENE TRANSFER IN CATTLE

Microinjection into pronuclei of ruminant zygotes is difficult because of the cytoplasmic yolk that obstructs visualization of pronuclei. In cattle oocytes centrifugation (13,000 U/min for 3 min) is required to concentrate the yolk material at one pole of ova and thereby allowing easier access to the pronuclei. This short-term centrifugation does not have detrimental effects on the developmental capacity of zygotes (Wall and Hawk, 1988). Centrifuged and microinjected zygotes were successfully cultured in rabbit oviducts and survived after transfer to cow uteri. Thus, injection of foreign DNA did not significantly increase embryonic losses within the initial two weeks after injection (Hawk et al., 1989). However, in summarizing the data obtained with gene transfer in cattle, it is obvious that high losses occur up to the blastocyst stage and after transfer prior to pregnancy establishment, that results in an average efficiency of 0.2% (Table 1). The application of the transgenic technology in cattle bears some distinct challenges which have allowed only a few laboratories to work with in vivo generated zygotes in the bovine. The main challenges include that the zygotes are difficult and expensive to obtain, the reproductive pattern is only one calf per pregnancy, the whole procedure is an expensive and time consuming undertaking, and finally the generation interval is very long, which prevents rapid progress in this area.

339
<table>
<thead>
<tr>
<th>Gene construct</th>
<th>Injected zygotes</th>
<th>Transferred zygotes</th>
<th>Recovery n (%)</th>
<th>Development n (%)</th>
<th>Fetuses/offspring n (%)</th>
<th>Transgenic n (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-TK</td>
<td>85</td>
<td>23</td>
<td>9 (39.1)</td>
<td>7 (77.8)(^a)</td>
<td>3 transfers, no pregnancies</td>
<td></td>
<td>Kraemer et al. (1985)</td>
</tr>
<tr>
<td>α-Fetoprotein</td>
<td>852</td>
<td>237</td>
<td>-</td>
<td>111 embryos</td>
<td></td>
<td>4 (0.5)</td>
<td>Church et al. (1986), McEvoy et al. (1987)</td>
</tr>
<tr>
<td>mMTrGH</td>
<td>47</td>
<td>47</td>
<td>-</td>
<td></td>
<td>4/17 pregnancies</td>
<td></td>
<td>Church (1987)</td>
</tr>
<tr>
<td>3 different constructs</td>
<td>1161</td>
<td>641</td>
<td>-</td>
<td>126 (10.9)(^b)</td>
<td>7 (0.6)</td>
<td></td>
<td>Biery et al. (1988)</td>
</tr>
<tr>
<td>RSVCAT</td>
<td>819</td>
<td>819</td>
<td>688 (84.0)</td>
<td>125 (18.2)</td>
<td>79 (9.6)</td>
<td>4 (0.5)</td>
<td>Roschla et al. (1989)</td>
</tr>
<tr>
<td>MT bGH</td>
<td>250</td>
<td>250</td>
<td>96 (38.4)</td>
<td>43 (44.8)</td>
<td>15 (6.0)</td>
<td>2 (0.8)</td>
<td>Krimpenfort et al. (1991)</td>
</tr>
<tr>
<td>CashLF</td>
<td>981(^d)</td>
<td>129</td>
<td>-</td>
<td></td>
<td>19 (1.9)</td>
<td>2 (0.2)</td>
<td></td>
</tr>
<tr>
<td>Various</td>
<td>3224</td>
<td>-</td>
<td>524 (16.2)</td>
<td>99 (3.1)</td>
<td>3 (0.1)</td>
<td></td>
<td>Hill et al. (1992)</td>
</tr>
<tr>
<td>IGF(_1)-constr.</td>
<td>7600(^f)</td>
<td>-</td>
<td>534 (7.0)</td>
<td>94 (1.2)</td>
<td>4 (0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15019</strong></td>
<td><strong>2146</strong></td>
<td><strong>793/1092 (72.6)</strong></td>
<td><strong>1233/11617 (10.6)</strong></td>
<td><strong>432/4269 (10.1)</strong></td>
<td><strong>26 (0.2)(^c)</strong></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) = 2-4 microinjected zygotes/recipient  
\(^{b}\) = Fetuses recovered and analysed at day 60  
\(^{c}\) = including one fetal monster  
\(^{d}\) = 981 out of 1154 in vitro produced zygotes survived microinjection  
\(^{e}\) = 19/4063 injected zygotes  
\(^{f}\) = in vitro produced zygotes
These problems make it extremely desirable to develop methods that permit the early detection of transgenesis in the early embryo to allow successful transfer of positive embryos into the uterus of appropriate recipients. We have injected a 6WTK-LacZ marker gene (4 µg/ml) either into the male pronucleus of in vitro produced zygotes, or into the cytoplasm as closely as possible to the metaphase plate of in vitro matured oocytes (Lemme et al., 1994). Following microinjection into the cytoplasm of 240 in vitro matured oocytes, two of them underwent lysis (0.8%), 162/238 (68.1%) showed evidence of cleavage (> 2 cells) and 45% (107/238) had developed to the 8- or 16-cell stage. In a total of 8 (3.4%) embryos LacZ-gene expression was detected and the majority (n=7) had developed to the 8- or 16-cell stage. Following microinjection into the male pronucleus of 184 zygotes, 24 (13%) underwent lysis, 76.3% (122/160) showed evidence of cleavage and 41.9% (67/160) had developed to the 8- or 16-cell stage. In 33 of the microinjected zygotes (20.6%), LacZ-gene expression was detected, of which 24 had developed to the 8- or 16-cell stage. This route of gene transfer was significantly (p < 0.05, chi square) more efficient than following injection into cytoplasm. Expression of the LacZ-gene was only rarely (2/40) observed in all blastomeres of an individual embryo. Following microinjection into the cytoplasm, 4/8 embryos expressed the LacZ gene in less than 50% of their blastomeres. In contrast, following microinjection into pronuclei, 58% of the embryos expressed the LacZ gene in less than 50% of their blastomeres, 30% of the embryos expressed LacZ exactly in 50% of their blastomeres and 12% expressed LacZ in more than half of their blastomeres. But only two embryos were seen with all the blastomeres being stained. Although a transient expression of the LacZ gene cannot be ruled out, the major finding of this study is that expression obviously only rarely occurs in all blastomeres of a given embryo. Furthermore, it confirms that microinjection into pronuclei is more efficient than into cytoplasm. Similarly, in mice it has been shown that the injected DNA was detected in only 44% of resulting morulae and only 26% of the blastocysts (Burdon and Wall, 1992). Injection of foreign DNA into the cytoplasm of cow and sheep metaphase II oocytes and pronucleus stage embryos showed an extensive degradation of the exogenous DNA (Powell et al., 1992).

Another powerful tool to detect the presence of foreign DNA is the Polymerase-Chain-Reaction (PCR). With the aid of PCR it was possible to show that only 6.5% of in vitro produced bovine blastocysts were positive for the microinjected DNA (Thomas et al., 1993). In another study, approximately 50% of bovine embryos could be eliminated prior to transfer using PCR (Horvat et al., 1993). However, the persistence of small quantities of microinjected foreign DNA in the embryo was considered to be responsible for the observation that embryos developing in vitro were in most cases negative a few weeks after placing them into culture in spite of PCR positive results obtained in biopsies shortly after microinjection (Bowen et al., 1993). These results clearly underline the difficulty to develop reliable procedures for the early detection of transgenesis which allow reliable distinction of positive from negative embryos prior to any transfer.

**GENE TRANSFER IN SHEEP**

The pronuclei in sheep zygotes can be visualized using Nomarski optics (differential interference contrast microscopy) at 200 fold magnification, which allows to observe pronuclei in more than 90% of the fertilized sheep zygotes. Generation of the appropriate pronucleus stage embryos requires a very strict reproductive management involving estrus synchronization and superovulation (see Halter et al., 1993). In the past, the number of offspring from microinjected zygotes usually did not exceed the 10% level with the proportion of transgenic animals being less than 1% (see Niemann et al., 1994). The majority of genes transferred into sheep have been growth hormone encoding gene constructs. However, the results were disappointing and in most cases without the desired effects in growth and feed efficiency. The elevated growth hormone levels frequently resulted in a clinical diabetic situation which led to an early death of the transgenic sheep (Rexroad et al., 1990). The results from the studies injecting growth regulating genes into ovine zygotes strongly indicate the need for a deeper understanding of regulation of growth and development prior to the
approach in transgenic animals. Similar to growth regulating genes, the approaches to modify wool production have thus far been unsuccessful to some extent. The two genes isolated from salmonella typhimurium encoding the two major enzymes responsible for cysteine synthesis were stably transfected into chinese hamster ovary cells and these cells showed an increased rate of cysteine biosynthesis (Sivaprasad et al., 1992). However, no positive effects of transgenes encoding these enzymes were observed in sheep wool growth patterns (Powell, 1994). Very recently transgenic sheep have been generated that express the visna virus envelope gene (Clements et al., 1994). In this study the gene construct consisting of the visna U3 region ligated to the envelope gene was microinjected into sheep zygotes to study the role of pathogenesis of lentivirus disease in sheep. Out of 368 zygotes transferred to 123 recipients 63 pregnancies (51%) were obtained and 3 out of 87 lambs were found to be transgenic carrying intact transgenes with both the U3 region and the envelope gene. All three transgenic lambs expressed the envelope glycoproteins, expression was observed in the differentiated macrophages in vitro, but not in monocytes. Lines from these transgenic sheep will provide a promising model to study whether the lentivirus glycoprotein prevents infection or modulates the disease after virus challenge (Clements et al., 1994).

Another promising area of the transgenic technology is the production of pharmaceutical proteins in the mammary gland of transgenic animals (see Clark et al., 1987; Halter et al., 1993; Niemann et al., 1994). The most prominent example for this is the recent observation that the milk of a transgenic sheep contains an average of 35 g/l A-1-Antitrypsin and the founder animal transmitted this remarkable trait to its offspring (Wright et al., 1991; Carver et al., 1993). Our work is aimed at expressing human coagulation factor VIII (FVIII) in the lactating mammary gland of sheep by using several different gene constructs mainly employing the whey acidic protein (WAP) or the β-lactoglobulin promotor elements to drive transcription of factor VIII cDNA (Niemann et al., 1992; 1993; Halter et al., 1993). In these experiments we have obtained a total of 9 transgenic offspring thus far with three different gene constructs (Table 2). The MT-FVIII transgenic animal showed a deletion in the transgene and did not transmit its trait to the offspring. The β-Lac FVIII transgenic male had not incorporated the transgene into the germ cells as shown by PCR-analysis of sperm-DNA while the female died soon after birth so that further analyses were not possible. Recently, we obtained another six transgenic animals using the β-Lac FVIII MT-I gene construct. These animals have reached puberty and the female is pregnant at the present time (February 1994) while two of the five surviving males have been mated to a group of females each, and semen analyses via PCR are currently underway. The latter construct harbours the introns of the metallothionein gene to overcome the problem of the potential insufficient expression of cDNA containing gene constructs (Brinster et al., 1988; Whitelaw et al., 1991). In parallel, we have produced transgenic mice bearing the various classes of constructs and are currently investigating expression of FVIII in the mammary gland of mice. The experiences gained from these studies have much improved our understanding of the methods to detect FVIII in body fluids and we have recently obtained evidence that FVIII is surprisingly stable for at least 10 hrs in sheep milk and can be determined with sufficient accuracy using specific ELISA or biological activity assays.

The efficiency of transgene expression is affected by various parameters (Westphal, 1989). First, it is affected by the position of the transgene in the genome (position effect) (Whitelaw et al., 1991); second it depends on the structure of the transgene, either genomic DNA or cDNA e.g. with or without introns (Brinster et al., 1988; Whitelaw et al., 1991), the promotor element, and/or the presence or absence of matrix attachment regions (MAR) (McKnight et al., 1992). We have recently generated gene constructs harbouring MARs and have finished a first series of microinjections and embryo transfers and are awaiting the birth of lambs in the summer 1994. Another novel approach to improve expression efficiency from large transgenes is the use of the cointegration technique (Clark et al., 1992). The strategy is to target the integration of the transgene into the genome in an active chromatin configuration by simultaneously transferring two gene constructs. Under these circumstances, one of the two genes known to be expressed in the target tissue is integrating via homologous recombination
and thereby inducing integration of the second transgene in a nearby position (Clark et al., 1992). This approach was successful, since expression efficiency of α-Antitrypsin cDNA and factor IX cDNA, both under control of the β-lactoglobulin promoter, were expressed significantly higher in transgenic mice as compared to controls derived from conventional microinjection.

In contrast to previous investigations (Ebert et al., 1991; Wright et al., 1991) we have consistently obtained high pregnancy rates that were to some extent related to the number of transferred zygotes per recipient. In the last season we have obtained pregnancy rates as high as 85% when transferring three zygotes per recipient (Table 3). This shows that the developmental capacity of zygotes is not impaired following microinjection with the various classes of our gene constructs. Because of the high percentage of multiplets the recipients need very intensive care to avoid any unwanted losses of progeny.

**TOWARDS IMPROVEMENTS OF GENE TRANSFER IN CATTLE AND SHEEP**

The recent emergence of the in vitro technology (IVM/IVF) will lead to an abundant availability of bovine zygotes (Gordon and Lu, 1990; Pavlok et al., 1992) and allows the widespread use of these zygotes for gene transfer purposes (Krimpenfort et al., 1991). A comparative analysis in vitro versus in vivo showed that with the current efficiency to obtain four calves with the in vitro procedure it is required to have 400 oocytes that will yield 300 injections leading to 27 blastocysts. On the other hand, the in vivo road would require 34 donors that yield 230 oocytes allowing 120 injections leading to 19 blastocysts and 4 calves. The average efficiency in both roads is 0.2% (Eyestone, 1994).

In addition, the recent advances in the embryonic stem (ES) cell (Saito et al., 1992; Strelchenko and Stice, 1994; Stice et al., 1994) and primordial germ cell (PGC) (Cherny and Merel, 1994; Stokes et al., 1994; Williams, 1994) technology make it likely that in the near future gene transfer will be used efficiently employing in vitro produced zygotes and either embryonic stem cell or primordial germ cell lines. This will allow to overcome position effects that affect expression efficiency.

Similar to cattle, the first priority in sheep is also to increase the efficiency of obtaining transgenic animals. As with cattle, embryonic stem cells could be instrumental in achieving this goal. In sheep, several ES like cell lines have been obtained (Notarianni et al., 1991; Tsuchiya et al., 1994). Cell lines derived from the ovine inner cell mass (ICM) have been injected into blastocysts, and subsequently transferred into recipients. However, final proof of germ line chimerism is still pending (Seamark, personal communication, 1994). It is also desirable to explore the technology of PGCs to have sufficient different cell lines available to study transgene integration and expression in cells prior to transfer into recipients. In contrast to cattle, in sheep the in vitro technology is not that far developed, and only few lambs have been born upon in vitro fertilization (Cheng et al., 1986; Crozet et al., 1987; Kelk et al., 1992). In sheep it has been determined that after a prolonged exposure to the in vitro system certain abnormalities become evident such as increased gestation length, increased birth weight and higher perinatal losses (Walker et al., 1992). The reasons for these abnormalities are unknown at present but need further investigations prior to any practical exploitation of the in vitro technology in both cattle and sheep. Nevertheless, the development of a functional in vitro system would be highly desirable for several reasons such as economic aspects and animal welfare. It would allow a more widespread application of these promising technologies with all the inherent potential benefits for a future-oriented animal production. The general availability of ES cells or PGCs would be a major breakthrough in terms of increasing efficiency in bovine and ovine gene transfer, both quantitatively and qualitatively.

**REFERENCES**


TABLE 2  EFFICIENCY OF MICROINJECTION FOR THE PRODUCTION OF FVIII TRANSGENIC SHEEP

<table>
<thead>
<tr>
<th>Gene constructs</th>
<th>MT-I-FVIII</th>
<th>β-lac-FVIII</th>
<th>β-lac-FVIII-MT-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. injected zygotes</td>
<td>130</td>
<td>613</td>
<td>336</td>
</tr>
<tr>
<td>no. transf. zygotes</td>
<td>57</td>
<td>475</td>
<td>219</td>
</tr>
<tr>
<td>no. recipients</td>
<td>21</td>
<td>112</td>
<td>106</td>
</tr>
<tr>
<td>pregnancy rate (%)</td>
<td>67</td>
<td>59</td>
<td>65</td>
</tr>
<tr>
<td>no. lambs born</td>
<td>21 (37%)</td>
<td>192 (40%)</td>
<td>103 (47%)</td>
</tr>
<tr>
<td>no. transgenic lambs</td>
<td>1 male</td>
<td>1 female</td>
<td>1 female</td>
</tr>
<tr>
<td>(%)</td>
<td>4.8% (1.7)</td>
<td>1.0% (0.4)</td>
<td>5.8% (2.7)</td>
</tr>
</tbody>
</table>

TABLE 3: PREGNANCY RATES RELATIVE TO THE NUMBER OF FVIII (BLACFVIII-MT I) MICROINJECTED OVINE ZYGOTES

<table>
<thead>
<tr>
<th>Transferred zygotes (n)</th>
<th>Recipients (n)</th>
<th>Pregnancies (n)</th>
<th>Multiplets (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td>(%)</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>34</td>
<td>21 (61.8)</td>
<td>33.3</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>22 (84.6)</td>
<td>31.8 (7 twins)</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>15 (78.9)</td>
<td>66.7 (7 twins, 3 triplets)</td>
</tr>
<tr>
<td>Total:</td>
<td>80</td>
<td>59 (73.8)</td>
<td>41.4 (24/58)</td>
</tr>
</tbody>
</table>

Season 92/93