# Effects of Time of Deoxyribonucleic Acid Microinjection on Gene Detection and In Vitro Development of Bovine Embryos

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## ABSTRACT

In vivo fertilized embryos were surgically collected from superovulated dairy cows to evaluate microinjection on embryo development and utilized the polymerase chain reaction technique for selection of transgenic embryos. Seventy-two percent of the embryos with visible pronuclei or nuclei were microinjected with DNA, and the remaining 28% served as uninjected controls. All embryos were cocultured with bovine oviductal epithelial cells. Mean final development scores of embryos within the same initial cell stage at collection were unaffected by microinjection. After 144 h of culture, 45% of the microinjected embryos developed to the morula or blastocyst stage. The transgene was detected in 50, 10, and 9% of demimorulae from embryos microinjected at the 1-, 2-, and 4-cell stages. Frequency of transgene detection was higher in morulae from 1-cell embryos than in morulae from 2- and 4-cell embryos. Use of in vitro coculture, embryo bisection, and polymerase chain reaction technique facilitated selection of bovine embryos that carried the transgene.

(**Key words**: deoxyribonucleic acid microinjection, bovine embryo culture)

**Abbreviation key**: **OEC** = oviductal epithelial cells, **PCR** = polymerase chain reaction.

## INTRODUCTION

Several investigators (5, 6, 23) interested in production of therapeutic human proteins in genetically engineered domestic animals have chosen the lactating mammary gland for transgene expression. Genes coding for therapeutic proteins have been successfully integrated and expressed in mammary glands of sheep (5, 23), goats (6), and pigs (21). Although milk production from these animals is copious, that of the dairy cow is superior; thus, the cow is an excellent choice for large-scale production of foreign proteins in the mammary gland.

Production of transgenic dairy cattle by pronuclear microinjection has been hampered by a need for large numbers of embryo recipients (13), increased embryo mortality rate following microinjection (12, 16), and low frequencies of transgene integration and expression (1, 13, 16). Efforts to reduce the number of embryo recipients required have included temporary placement of the microinjected embryos into rabbit, ovine, or bovine oviducts (1, 9, 16) or culturing of the embryos in vitro (12) to facilitate selection of only those embryos that develop to the morula stage and to eliminate the need for surgical transfer of oviductal stage embryos.

Increasing frequencies of transgene integration might enhance production of transgenic cattle; however, current lack of information on mechanisms of transgene integration requires researchers to use other techniques to increase the number of transgenic calves born per transfer. Ninomiya et al. (14) used the polymerase chain reaction (**PCR**) technique (18) for detection of a transgene in murine demimorulae produced from matings with transgenic males that carried two copies of the transgene. King and Wall (11) reported that a single-copy gene encoding the $\beta$ chain of bovine LH could be detected in purified bovine genomic DNA representing 25 cells. We recently reported (19)

Received June 3, 1993.
Accepted October 18, 1993.
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that either blastomere biopsy or embryo bisection technique was suitable for cell recovery for DNA amplification.

The objectives of this study were to evaluate the effect of DNA microinjection on bovine embryo development and to utilize in vitro culture, embryo bisection, and PCR to facilitate selection of transgenic bovine embryos for subsequent transfer.

## MATERIALS AND METHODS

### Superovulation

Twenty-four reproductively healthy, nonlactating dairy cows were superovulated with 32 mg of FSH (Schering Corp., Kenilworth, NJ) using a decreasing dose regimen over 4 consecutive d initiated between d 9 and 13 of the estrous cycle. The $PGF_{2\alpha}$ (Lutalyse®; Upjohn, Kalamazoo, MI) was administered 72 h (30 mg) and 84 h (10 mg) after the initial FSH injection to induce luteal regression. Cattle displayed estrus 36 to 48 h after the first $PGF_{2\alpha}$ injection and were bred by natural service at 0 h and by AI 12 and 24 h after the first observation of estrus.

### Embryo Recovery

The 1- to 4-cell bovine embryos were surgically collected 36 to 64 h after the first observation of estrus. Oviducts were exteriorized via the caudal flank approach described by Wolfe et al. (22). Anesthesia was initiated with glycerol guaifenesin and thiopental and maintained with halothane. After initiation of anesthesia, the cows were positioned in lateral recumbency. The rear limb of the exposed side was pulled caudally, and the flank of the same side was prepared and draped for aseptic surgery.

The proximal ovary, oviduct, and uterine horn were exteriorized by gentle traction on the broad ligament. The number of ovulations and follicles was recorded. A polyethylene cannula (polyethylene tubing, i.d. 1.14 mm, o.d. 1.57 mm; Intramedic®; Clay Adams, Parsippany, NJ) was threaded through the ostium of the infundibulum and 4 to 5 cm into the ampulla of the oviduct to facilitate fluid collection. Approximately 20 to 30 ml of PBS (embryo transfer freezing medium; Gibco, Grand Island, NY) were flushed through the oviduct by introducing a 20-gauge blunt-ended needle into the uterine lumen 1 cm from the uterotubal junction. The fluid was collected into sterile test tubes and transported to the laboratory at 37°C. This procedure was repeated with the contralateral oviduct when it could be exteriorized through the same incision site. The reproductive tract was irrigated with 3% (vol/vol) glycerol in sterile saline to reduce adhesion formation before return to the abdominal cavity (2). When the distal ovary could not be exteriorized through the first incision site, the cow was rotated, and the entire procedure described was repeated to flush the remaining oviduct.

Recovered fluid was searched for embryos and for oviductal epitheleal cells (**OEC**). The OEC were washed with tissue culture medium 199 with Earle's salts (Gibco) supplemented with 10% newborn calf serum (Gibco), pyruvate (1 mg/ml), penicillin (1 mg/ml), and streptomycin (1 mg/ml) 4 to 6 times in a conical tube. After being washed, OEC were placed in 25-$\mu$l drops of tissue culture medium 199 and 10% newborn calf serum and maintained at 39°C in an atmosphere of 5% $CO_2$ in air.

### Embryo Microinjection

Recovered embryos were washed three times in PBS and transferred in 500 $\mu$l of medium to sterile plastic vials for centrifugation. Embryos were centrifuged at 16,000 × *g* for 6 min to facilitate visualization of pronuclei and nuclei. Following centrifugation, embryos were observed at 200× under Hoffman modulation optics. Seventy-two percent of the embryos with visible nuclei or pronuclei were microinjected, and the remaining embryos were assigned to the control treatment. A greater proportion of embryos was assigned to the microinjection treatment to optimize the number of potentially transgenic embryos.

To perform microinjection, the embryo was held by suction with a holding pipette (30 $\mu$m i.d.) controlled by one arm of the micromanipulator. The other arm controlled the injection pipette, which was connected to an Eppendorf automatic microinjector (model 5242; Eastern Microscopes, Raleigh, NC). The DNA used for microinjection was a 5.7-kb fragment consisting of the mouse whey acidic

protein promoter and 3′ elements with a human protein C cDNA inserted at the whey acidic protein coding region (4, 7, 21). The DNA was diluted in a buffer of 10 $\mu M$ Tris·HCl-1 and 8 m$M$ EDTA, pH 8.0, to make a final DNA concentration of 250 copies per picoliter. The most visible or largest pronucleus or nucleus was injected with 1 to 2 pl of the DNA solution. One nucleus of each 2-cell embryo and two nuclei of 4-cell embryos were microinjected to maintain consistency to allow for possible DNA integration into one-half of the genome. Visualization of swelling of the pronucleus or nucleus indicated a successful injection.

#### Embryo Culture

Microinjected embryos were cultured for approximately 144 h in 25-$\mu$l drops of tissue culture medium 199 and 10% newborn calf serum, pyruvate, penicillin, streptomycin, and OEC in an atmosphere of 5% $CO_2$ in air at 39°C. Morphological stage of development was assessed 48 h after the initiation of culture. All OEC and 4- and 8-cell embryos were washed after 48 h of culture and returned to tissue culture medium 199 supplemented with 10% newborn calf serum, pyruvate, penicillin, and streptomycin. Morphological development was assessed at the termination of culture.

#### Amplification of the Transgene

Microinjected embryos developing to the morula stage in culture were bisected to obtain cellular material for amplification of the transgene by PCR. Bisection occurred in a chamber consisting of a 100- × 15-mm sterile, plastic Petri dish lid that contained a 100-$\mu$l microdrop of PBS covered with silicone oil. A microsurgical blade (Special Blade S; Storz, Heidelberg, Germany) was passed vertically through the zona pellucida and cell mass. One demi-embryo was transferred with <2 $\mu$l of PBS to 5 $\mu$l of filtered-sterilized (.22 $\mu M$) deionized water in a sterile, 250-$\mu$l plastic vial. Vials were held at −86°C and thawed immediately prior to PCR.

The PCR was performed as described by Saiki et al. (17) using primers 5′-CTCCTGCAGTGTCACCCCGCAGT-3′ and 5′-CACCATGTTGCTCATGACCTCGCTG-3′, which are complementary to the human protein C gene. Primers were positioned at 514 and 1143 of protein C beginning at the 5′ end. The predicted size of the PCR product is 630 bp. Demi-embryos were subjected to 40 cycles of PCR in a volume of 25 $\mu$l that contained 50 m$M$ KCl; 10 m$M$ Tris (pH 8.8); 1.5 m$M$ $MgCl_2$; .1% Triton X-100; .2 $\mu M$ each 2′-deoxyadenosine 5′-triphosphate, 2′-deoxythymidine 5′-triphosphate, 2′-deoxycytidine 5′-triphosphate, and 2′-deoxyguanosine 5′-triphosphate; .5 $\mu M$ of each oligonucleotide primer; and .625 units of *Taq* polymerase (*Thermus aquaticus*; Promega, Madison, WI). The entire reaction mixture was overlaid with 25 $\mu$l of paraffin oil. Each cycle consisted of denaturation at 96°C for 15 s, annealing for 120 s at 55°C, and extension of primers for 30 s at 77°C. The initial cycle had denatured DNA by heating of the samples to 96°C for 1 min. Reaction products were visualized by addition of 10 $\mu$l of 6× loading dye (15% Ficoll®-400, .25 $\mu$l bromophenol blue; Sigma Chemical Co., St. Louis, MO) to each tube and loading 25 $\mu$l of this mixture in a 1% agarose gel (SeaKem®; FMC BioProducts, Rockland, ME) containing .5 $\mu$g/ml of ethidium bromide. Amplified DNA was subjected to electrophoresis in a base of 40 m$M$ Tris, 40 m$M$ acetate, 2 m$M$ EDTA, and .5 $\mu$g/ml of ethidium bromide for 1 h at 50 V. Amplified PCR fragments were visualized by UV transillumination and photography.

The remaining demi-embryos were returned to the coculture system immediately after microsurgery. The DNA amplification and identification of samples containing the transgene via agarose gel electrophoresis was completed approximately 7.5 h after microsurgery.

#### Embryo Transfer

Embryos identified as positive for the transgene were transferred to recipient heifers within 10 h after microsurgery. Heifers were synchronized with 35 mg of $PGF_{2\alpha}$ 12 to 24 h after $PGF_{2\alpha}$ was administered to the superovulated donor.

Embryos were loaded into a .25-cc straw and transferred nonsurgically to the uterus with a .25-cc, 8.27-cm Cuozzo Gun (EmCal, San Diego, CA). Following transfer, heifers were observed for signs of repeat estrus, and preg-

nancies were confirmed by rectal palpation at approximately 35 d of gestation.

### Statistical Analysis

Mean final development scores of embryos for each treatment were calculated, and analysis was performed by ANOVA. Initial cell stage and microinjection treatment were independent variables in the model. Differences in percentage of embryos developing to the morula stage for microinjection, initial cell stage, and frequency of transgene detection were tested by chi-square analysis.

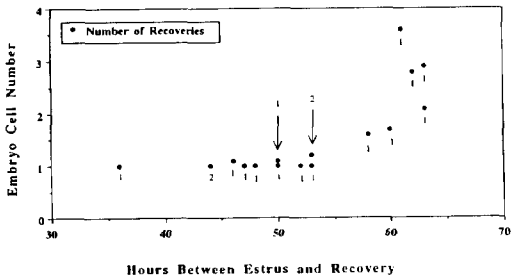


Figure 1. Mean cell number in embryos collected between 36 and 63 h after first observation of estrus. ● = The mean cell number of embryos recovered; n = the number of cows collected at each time.

## RESULTS

Twenty-two of the 24 superovulated cows had 18.5 ± 3.9 ($\overline{X}$ ± SE) corpora hemorrhagica with 14.9 ± 2.7 embryos recovered. Two cows failed to ovulate prior to surgery. Embryo data from two cows that subsequently had only unfertilized ova were included in the embryo recovery estimate. Embryo recovery rate, determined by the total number of embryos recovered and corpora hemorrhagica observed, was 80.4% (329 per 409). Of the 329 embryos recovered, 62 (19%) were at the 4-cell stage, 57 (17%) were classified as 2-cell, 208 (63%) were 1-cell, and 2 (1%) were degenerate. Fifty-three of the 208 1-cell ova (25%) were classified as unfertilized because of lack of visible pronuclei and failure to cleave in culture. No attempt was made to stain pronuclei in the uncleaved 1-cell ova.

Stage of embryo development at recovery was affected by time between the first observation of estrus and surgery ($P < .01$; Figure 1). Two donors collected at 48 and 46 h postestrus had only unfertilized ova and were not included in Figure 1. Ninety-four percent (123 embryos) of the 131 embryos collected at 44 to 53 h after first observation of estrus were at the 1-cell stage; 6 embryos (5%) were at the 2-cell stage, and 2 embryos (1%) were unfertilized ova. The earliest recovery time for collection of fertilized 1-cell embryos was 36 h after first observation of estrus. When the interval between first observation of estrus and surgery was increased to ≥60 h, the recovered embryo population was primarily at the 2- to 4-cell stages.

A total of 105 1-cell, 49 2-cell, and 43 4-cell embryos were microinjected with whey acidic protein-human protein C; 50 1-cell, 8 2-cell, and 19 4-cell embryos remained uninjected. Five (3%) 1-cell embryos were destroyed by DNA microinjection. The percentage of embryos with at least one cleavage after the initial 48 h of culture and the percentage of embryos developing to the morula stage were unaffected by initial cell stage or microinjection ($P > .05$; Table 1).

The mean final development score of embryos that cleaved at least once in culture was

TABLE 1. Development of 1-, 2-, and 4-cell injected and uninjected embryos cultured in vitro.

| Initial cell stage | Treatment | n | Cleaved after 48 h | | Developing to morula | |
|---|---|---|---|---|---|---|
| | | | (n) | (%) | (n) | (%) |
| 1 | Injected | 100 | 88 | 88 | 42 | 42 |
| | Uninjected | 50 | 50 | 100 | 15 | 30 |
| 2 | Injected | 49 | 45 | 92 | 18 | 37 |
| | Uninjected | 8 | 8 | 100 | 5 | 63 |
| 4 | Injected | 43 | 35 | 81 | 27 | 63 |
| | Uninjected | 19 | 19 | 100 | 8 | 42 |

affected ($P < .05$) by the initial cell stage at recovery and the microinjection by initial cell stage at recovery interaction (Table 2). Uninjected 4- and 2-cell embryos had higher ($P < .05$) mean development scores than did uninjected 1-cell embryos, but mean development scores for injected 4- and 2-cell embryos did not differ from that of injected 1-cell embryos. Mean development scores for injected 1-, 2-, and 4-cell embryos were not different ($P > .05$) from those of uninjected 1-, 2-, and 4-cell embryos.

A total of 87 microinjected 1-, 2-, and 4-cell embryos developed to the morula stage. Sixty-five morulae (75%) were considered to be acceptable for bisection (Table 3). Morulae that had numerous blastomeres that degenerated or failed to compact were considered to be unacceptable for bisection. Frequency of transgene detection was greater ($P < .01$) in morulae that developed from embryos microinjected at the 1-cell stage than from embryos microinjected at the 2- and 4-cell stages.

Thirteen of the 19 demi-embryos containing whey acidic protein-human protein C were transferred to six synchronized recipients. Three recipients returned to estrus with no evidence of an extended estrous cycle. One recipient had a 28-d estrous cycle. A membranous sac was expelled from one recipient 36 d after embryo transfer; the whey acidic protein-human protein C gene was detected in DNA extracted from the recovered membrane. The remaining recipient was confirmed pregnant 45 d after embryo transfer but was observed to be in estrus 51 d posttransfer. The fetus was not recovered from this recipient.

## DISCUSSION

The recovery of 2- and 4-cell embryos during collection of in vivo fertilized 1-cell embryos represents one of the many problems encountered in attempts to produce transgenic cattle by pronuclear DNA injection. Brinster et al. (3) reported that pronuclear microinjection produced transgenic mice more efficiently than DNA microinjection into nuclei of more advanced embryos. These results have caused researchers to enhance production of bovine 1-cell embryos by determination of the optimal time between estrus and collection (13, 16) or by production of 1-cell embryos by in vitro fertilization (13). Embryo recovery between 44 and 53 h after the onset of estrus was optimal for collection of 1-cell fertilized embryos (Figure 1). Collection at 36 to 48 h postestrus in an earlier study (20) yielded a higher proportion of unfertilized ova (50%) than in the present study (17%).

The percentage of microinjected 1-, 2-, and 4-cell bovine embryos developing to the morula stage in vitro was unaffected by initial cell stage (Table 1). Hawk et al. (9) reported that 55% of 120 bovine microinjected embryos recovered from rabbit oviducts had developed to the morula stage. The proportion of microinjected 1-cell embryos developing to the morula stage in vitro (42%) was comparable with the results of Hawk et al. (9) and higher than the 21% of microinjected 1-cell embryos developing to the morula stage in the sheep oviduct reported by Biery et al. (1).

The proportion of 1-, 2-, and 4-cell embryos developing to the morula stage in vitro was unaffected by microinjection (Table 1). Hawk et al. (9) reported similar results with in vivo

TABLE 2. Least squares mean final development scores for injected and uninjected 1-, 2-, and 4-cell embryos cleaving at least once in vitro.

| Initial cell stage | Final development score[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Injected | | | Uninjected | | | All | | |
| | $\bar{X}$ | SE | (n) | $\bar{X}$ | SE | (n) | $\bar{X}$ | SE | (n) |
| 1 | 5.1 | .2[ab] | 88 | 4.4 | .3[b] | 50 | 4.8 | .2[a] | 138 |
| 2 | 4.6 | .3[b] | 45 | 5.9 | .6[a] | 8 | 5.2 | .3[ab] | 53 |
| 4 | 5.6 | .3[a] | 35 | 5.6 | .4[a] | 19 | 5.6 | .2[b] | 54 |

[a,b]Development scores with different superscripts within the same column differ ($P < .05$).

[1]Development was scored as 1 = 1-cell, 2 = 2-cell, 3 = 4-cell, 4 = 8-cell, 5 = 16-cell, 6 = morula, 7 = early blastocyst, 8 = blastocyst, and 9 = expanded blastocyst.

TABLE 3. Transgene detection in demimorulae derived from microinjected 1-, 2-, and 4-cell bovine embryos.

| Initial cell stage | Developing to morula | Morulae acceptable for bisection | | Transgene detection | |
|---|---|---|---|---|---|
| | (n) | (n) | (%) | (n) | (%) |
| 1 | 42 | 32 | 76 | 16 | 50[a] |
| 2 | 18 | 10 | 56 | 1 | 10[b] |
| 4 | 27 | 23 | 85 | 2 | 9[b] |
| Total | 87 | 65 | 75 | 19 | 29 |

[a,b]Chi-square ($P < .01$) between initial cell stages.

fertilized, microinjected bovine embryos cultured in rabbit oviducts. In contrast, culture of in vitro fertilized, microinjected bovine embryos decreased the percentage of embryos developing to the morula stage following microinjection (8, 12). The results in this report and those of Hawk et al. (9) and Hill et al. (10) suggest that embryos fertilized in vivo are better candidates for DNA microinjection and culture than are embryos fertilized in vitro.

Mean final development score of the 1-, 2-, and 4-cell bovine embryos was unaffected by microinjection but differed among initial cell stages (Table 2). Mean final development score for all 4-cell embryos was higher than for 1-cell embryos but was not different from 2-cell embryos. However, microinjected 1- and 4-cell embryos had similar mean final development scores. These results are contrary to those of an earlier study by Brinster et al. (3), in which microinjection of 1- and 2-cell mouse embryos led to lower postmicroinjection survival rates and integration for the embryos injected at the 2-cell stage. Data presented by Pursel et al. (15) and in our study suggest that developmentally advanced livestock embryos (>1-cell) are more resilient to microinjection than advanced mouse embryos.

Sixty-five morulae (75%) of the 87 morulae that developed from microinjected 1-, 2-, and 4-cell embryos were considered to be acceptable for bisection (Table 3). Transgene detection rate was higher in morulae that developed from embryos injected at the 1-cell stage (16 of 32) than from embryos injected at the 2-cell (1 of 10) or 4-cell (2 of 23) stages. Microinjection and subsequent analysis of half of the cells in the 2- and 4-cell embryos may have reduced transgene detection in morulae developing from embryos microinjected at the 2- and 4-cell stages. We are not aware of any other studies that have evaluated gene integration rates in embryos that developed from bovine embryos microinjected at different cell stages.

Comparable percentages of microinjected 1-, 2-, and 4-cell embryos developed to the morula stage in vitro. Frequency of transgene detection was higher in morulae that developed from microinjected 1-cell embryos. These results indicate that the pronucleus is the preferred target for DNA microinjection.

Although low numbers of transferable transgene-positive demi-embryos did not allow establishment of pregnancy status, the combination of in vitro culture, embryo bisection, and DNA amplification by PCR can be utilized for selection of potentially positive transgenic bovine embryos for nonsurgical transfer to synchronized recipients.

## ACKNOWLEDGMENTS

This research was supported by a grant from the Virginia Agricultural Council.

## REFERENCES

1 Biery, K. A., K. R. Bondioli, and F. J. De Mayo. 1988. Gene transfer by pronuclear injection in the bovine. Theriogenology 29:224.(Abstr.)

2 Bondurant, R. H. 1986. Page 63 *in* Current Therapy in Theriogenology. D. A. Morrow, ed. W. B. Saunders Co., Philadelphia, PA.

3 Brinster, R. L., H. Y. Chen, M. E. Trumbauer, M. K. Yagle, and R. D. Palmiter. 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Natl. Acad. Sci. USA 82: 4438.

4 Campbell, S. M., J. M. Rosen, L. G. Hennighausen, U. Strechjurk, and A. E. Sippel. 1984. Comparison of the

whey acidic protein genes of the rat and mouse. Nucleic Acids Res. 12:8685.
5 Clark, A., H. Bessos, J. Bishop, P. Brown, S. Harris, R. Lathe, M. McClenaghan, C. Prowse, J. Simons, C. Whitelaw, and I. Wilmut. 1989. Expression of human anti-hemophilic factor IX in the milk of transgenic sheep. Bio/Technology 7:487.
6 Denman, J., M. Hayes, C. O'Day, T. Edmunds, C. Bartlett, S. Hirani, K. M. Ebert, K. Gordon, and J. M. McPherson. 1991. Transgenic expression of a variant of human tissue-type plasminogen activator in goat milk: purification and characterization of the recombinant enzyme. Bio/Technology 9:839.
7 Foster, D. C., S. Yoshitake, and E. W. Davie. 1985. The nucleotide sequence of the gene for human protein C. Proc. Natl. Acad. Sci. USA 82:4673.
8 Gagne, M., F. Pothier, and M. A. Sirard. 1990. Developmental potential of early bovine zygotes submitted to centrifugation and microinjection following in vitro maturation of oocytes. Theriogenology 34:417.
9 Hawk, H. W., R. J. Wall, and H. H. Conley. 1989. Survival of DNA-injected cow embryos temporarily cultured in rabbit oviducts. Theriogenology 32:243.
10 Hill, K. G., J. Curry, F. J. DeMayo, K. Jones-Diller, J. R. Slapak, and K. R. Bondioli. 1992. Production of transgenic cattle by pronuclear injection. Theriogenology 37:222.(Abstr.)
11 King, D., and R. J. Wall. 1988. Identification of specific gene sequences in preimplantation embryos by genomic amplification: detection of a transgene. Mol. Reprod. Dev. 1:57.
12 Krimpenfort, P., A. Rademakers, W. Eyestone, A. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. E. Platenburg, F. Pieper, R. Strijker, and H. de Boer. 1991. Generation of transgenic dairy cattle using 'in vitro' embryo production. Bio/Technology 9: 844.
13 McEvoy, T. G., and J. M. Sreenan. 1990. The efficiency of production, centrifugation, microinjection and transfer of one- and two-cell bovine ova in a gene transfer program. Theriogenology 33:819.
14 Ninomiya, T., M. Hoshi, A. Mizuno, M. Nagao, and A. Yuki. 1990. Selection of transgenic preimplantation embryos by PCR. J. Reprod. Fertil. Suppl. 41: 222.(Abstr.)
15 Pursel, V. G., K. F. Miller, C. A. Pinkert, R. D. Palmiter, and R. L. Brinster. 1988. Effect of ovum cleavage stage at microinjection on embryonic survival and gene integration in pigs. Page 480 *in* Brief Comm. 11th Int. Congr. Anim. Reprod. AI. Dublin, Ireland.
16 Roschlau, K., P. Rommel, L. Andreewa, M. Zackle, D. Roschlau, B. Zackle, M. Schwerin, R. Huhn, and K. G. Gazarjan. 1989. Gene transfer experiments in cattle. J. Reprod. Fertil. Suppl. 38:153.
17 Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science (Washington, DC) 239:487.
18 Saiki, R. K., S. J. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of $\beta$-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science (Washington, DC) 230: 1350.
19 Sparks, A.E.T., R. S. Canseco, and F. C. Gwazdauskas. 1991. Evaluation of bovine morula bisection and biopsy on subsequent embryonic development in vitro. Page 332 *in* Preimplantation Embryo Development. B. D. Bavister, ed. Serono Symposia, USA. Springer-Verlag, New York, NY.
20 Sparks, A.E.T., F. C. Gwazdauskas, and M. L. McGilliard. 1992. Culture of one-cell bovine embryos in explanted mouse oviduct and bovine oviductal epithelial cells. Theriogenology 37:587.
21 Velander, W. H., J. L. Johnson, R. L. Page, C. G. Russel, A. Sabramanian, T. D. Wilkins, F. C. Gwazdauskas, C. Pittius, and W. D. Drohan. 1992. High level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. Proc. Natl. Acad. Sci. USA 89:12003.
22 Wolfe, D. F., M. G. Riddell, P. W. Mysinger, R. L. Stringfellow, R. L. Carson, and P. D. Garrett. 1990. A caudal flank approach for the collection of oviductal-stage bovine embryos. Theriogenology 34:167.
23 Wright, G., A. Carver, D. Cottom, D. Reeves, A. Scott, P. Simons, I. Wilmut, I. Garner, and A. Colman. 1991. High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. Bio/Technology 9:830.