Future aspects of micromanipualtion with embryos for cattle breeding

H. Kräusslich and G. Brem
Lehrstuhl für Tierzucht, L.M. Universitat, Munchen

Embryo micromanipulation techniques and their potential genetic impact in dairy cattle are discussed. In addition, some aspects of gene transfer are mentioned. Only the technique of splitting bovine embryos and the subsequent transfer of half-embryos has reached a stage which might make its application to cattle breeding possible. However, the main use of identical twins produced by micromanipulation is expected to lie in genetic research. A better understanding of the interaction of maternal effects and genetic effects may influence genetic theories and subsequently animal breeding plans. Identical pairs of young bulls would allow characteristics to be evaluated by means which are incompatible with the later use of the bulls for breeding.
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Embrio mikromanipulasietegnieke en hul potensiële genetiese inslag by melkbeeste word bespreek. Aspekte van geenoorplasing word ook genoem. Slegs die tegniek vir die splitsing van beesembrios en die daaropvolgende oorplasing van half-embrios het tot dusver 'n stadium bereik wat die toepassing daarvan in die teelt van beeste moontlik mag maak. Dit word verwag dat genetiese navorsing die belangrikste gebruikswyse vir identiese tweelinge, deur makro-manipulasie geproduseer, sal wees. 'n Beter begrip van die interaksie tussen maternale en genetiese effekte mag genetiese teorië en gevolglik ook diereteeltplanne beïnvloed. Die beskikbaarheid van jong bulle, in identiese pare, sal tot gevolg hê dat eienskappe evalueer kan word deur tegnieke wat onaanvaarbaar mag wees vir die latere gebruik van die bulle tydens teling.
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H. Krausslich* and G. Brem
Lehrstuhl für Tierzucht, LM Universiteit Munchen,
Veterinärstrasse 13, 8 München 22, F.R West Germany

*To whom correspondence should be addressed.

Introduction

Man has been interested in altering the genetic make-up of higher animals for thousands of years, dating back to the first primitive attempts at crossing 'good' animals with other 'good' animals. The reasons for making these alterations were in the main practical but occasionally fanciful.

Artificial insemination has accelerated the improvement of animal breeding considerably. Techniques such as superovulation, storage of embryos *in vitro* and embryo transfer were developed and applied to animal breeding to further increase the effectiveness of selection programmes.

New techniques, such as microsurgery, micromanipulation and gene transfer in the embryo, developed within the last five years, might enable us for the first time to modify the genetic composition of an animal by means other than standard breeding techniques. The aim of this paper is to review the recent research on these new techniques and their practical applications to livestock improvement and animal breeding.

Microsurgery in cattle

The pre-implantation embryo is used for micromanipulation. The technique of embryo transfer (ET) is indispensable for the production of living animals from micromanipulated embryos. Experiments using microsurgery on domestic animals currently aim at the production of identical twins, multiplets and chimeras.

Identical twins

Identical twins can be produced by splitting embryos up to day six and subsequently transfering the halves to synchronous recipients. Precompaction embryos at the two, four or eight cell stage have to be collected surgically. After division, it is necessary to put each half into a zona pellucida or surrogate zona pellucida made of agar (Willadsen, 1979). Postcompaction embryos can be collected surgically or non-surgically.

In our experiments postcompaction embryos used for splitting are collected non-surgically from superovulated donor cows on day six after insemination. The collected embryos are placed into a dish with Phosphate Buffered Saline Medium (PBS) and Foetal Calf Serum (FCS) and manipulated under a stereomicroscope (100 × enlargement). Only compacted morulae (Figure 1) classified as excellent should be taken for splitting experiments.

Figure 2 shows the splitting procedure schematically. The zona pellucida is cut with the sharp edge of a razor blade (5 μm) as shown in Figure 3. Then the embryo is split into two approximately equal halves with a very fine knife or glass

needle (Figure 4). One of the halves is removed from the zona and put in a foreign zona (Figure 5 and 6). Our experience shows that it is best to transfer the half-embryos within a few hours of splitting. However, it is reported in the literature that half-embryos can be cultured or frozen (Lehn-Jensen, H., & S.M. Willadsen, 1983). An experienced and skilled person requires 10 to 15 minutes to split an embryo. Transfer can be carried out non-surgically or surgically. Pregnancy rates vary between 8 % and 75 % per half embryo (Table 1a). Pregnancy rates depend on the preselection of the embryos. In some experiments all embryos considered transferable were used for splitting (Table 1b). In other experiments only the best embryos were selected.

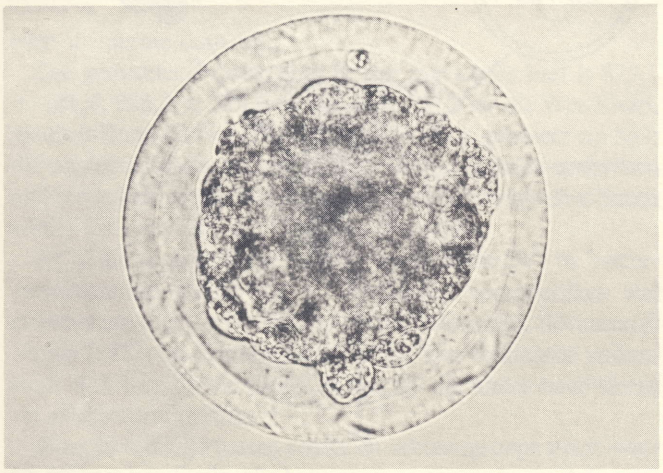

Figure 1 Compacted morula of a cow.

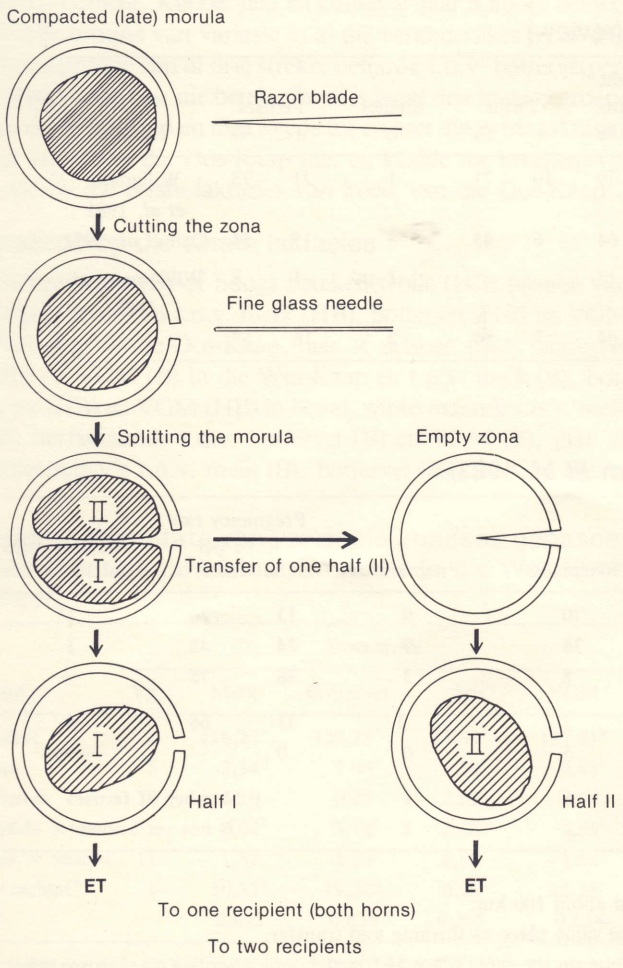

Figure 2 Scheme of splitting embryos.

Chimeras

Producing chimeras is an experimental technique which is in many ways the opposite of cutting embryos in to halves. The zona pellucidae are removed from two (sometimes more) preimplantation embryos which are then aggregated within one

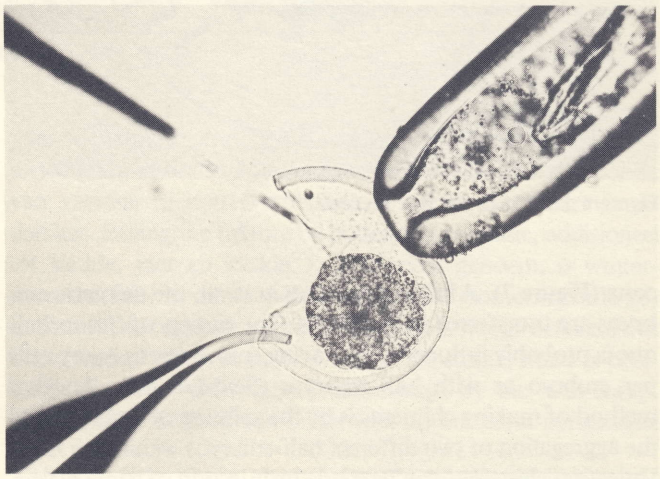

Figure 3 Morula from Figure 2 with opened zona.

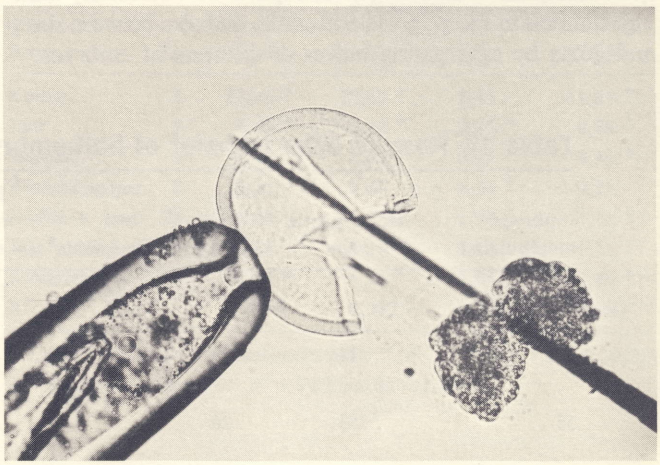

Figure 4 Morula from Figure 2 split in two halves.

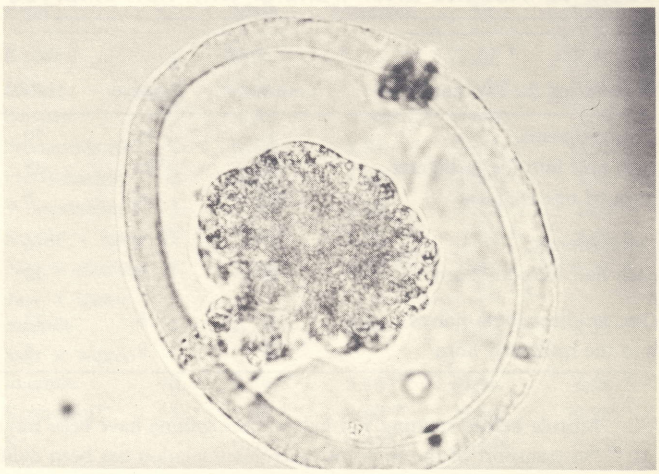

Figure 5 Half I in its own zona.

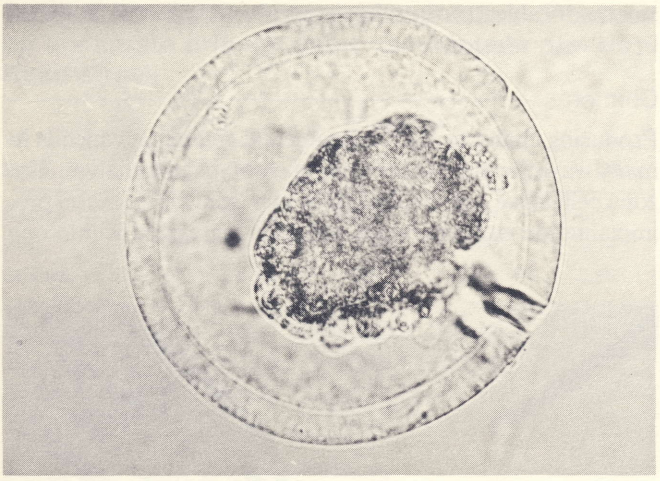

Figure 6 Half II in the foreign zona.

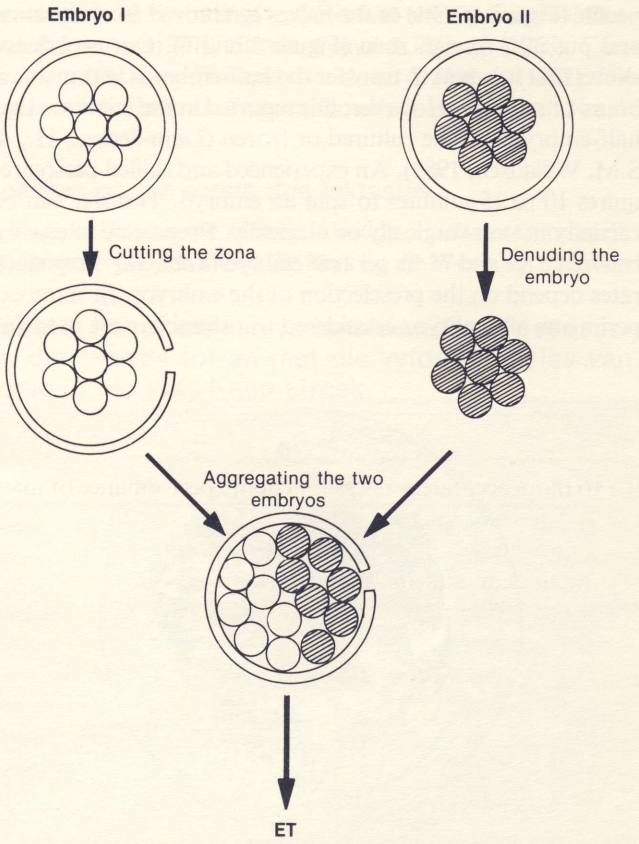

Figure 7 Scheme for producing chimeras by microsurgery.

zona (Figure 7). After compaction *in vitro*, the chimeric embryos are transferred to recipients. The success of this technique is probably influenced by having four times as many cells per embryo as with half-embryos (Seidel, 1982). Another method of making chimeras is by the splitting of embryos and the aggregation of two different half-embryos within one zona. 'Injection chimeras' can be made by injection of some blastomeres of one morula into the innner-cell-mass of another embryo. The relative contributions of the embryos are then usually quite unequal. Willadsen (1982) reports on succesfully making chimeras in sheep, goats and cattle, and also between sheep and goats by aggregating halves or quarters of embryos.

Table 1a Results after transfer of half-embryos (review)

Embryos manipulated	Monozygotic pairs	Pairs to ET	Recipients[a]	Pregnant recipients n	Pregnant recipients %	Twins n	Twins %	Singles n	Fetuses n	Fetuses %	Author
		14	14 s	11	79	10	71	1	21	75	Willadsen *et al.* 1981
16	14	14	14 ns	9	64	6	43	3	15	54	Ozil *et al.* 1982
			6 ns	1	17			1	1	8	Williams *et al.* 1982
55	53	20									
			14 s	9	64	7	50	2	16	57	

[a] s: surgical transfer; ns: non-surgical transfer

Table 1b Results after transfer with half-embryos (Brem *et al.* 1983)

Transfer of the two halves to	Transport[a]	Embryo	Halves	Recipients	Pregnancies	Pregnancy rate in % per halfembryo		Monozygotic pairs of twins
Two recipients,	I	35	70	70	9	13	26	1[b]
each half into the ipsilateral	II	19	38	38	9	24	48	1
horn of one recipient	III	4	8	8	3	38	75	–
One recipient,	I	3	6	3	2	33	66	1
each half into one horn	II	5	10	5	0	0	–	–
One recipient, both halves	I	6	12	6	1	Number of fetuses		
into the ipsilateral horn	II	2	4	2	1	is not yet known		

[a] I: Morula before splitting and halves after spliting have been transported about 100 km.
II: No transport of the embryos, micromanipulation has been done on the same place as flushing and transfer.
III: Only the morula has been transported, micromanipulation has been done on the same place as transfer.

[b] One fetus aborted after a 50-day pregnancy.

In-vitro fertilization in cattle

A procedure for bovine *in vitro* fertilization (IVF) involving recovery of oocytes near the time of ovulation, fertilization with *in vitro* capacitated sperm, embryoculture and transfer has led to the development of normal offspring. Brackett (1983) and Brackett, Seidel & Seidel (1981) reported the birth of one calf following IVF.

Additional research is needed to facilitate recovery of nature oocytes and to improve *in vitro* conditions to insure development of viable embryos that can continue to develop normally following non-surgical uterine transfer. Bovine IVF is a promising technique for the following purposes:

(a) to overcome certain types of infertility,
(b) to produce large numbers of half-siblings simultaneously,
(c) to greatly extend valuable semen,
(d) to more accurately assess functional performance of male and female gametes,
(e) to provide synchronously developing pronuclear stage ova for nuclear transfer and/or gene injection.

Other possibilities in research and for future applications may arise.

Sexing

Shapley (1983) reported the birth of two female calves the sexes of which were determined before embryo transfer. Monoclonal A-Y-antigenes were used for sexing. This method was patented for the Genetic Engineering Company in Denver Colorado. Thus, sex determination of embryos will become routine in the near future. This method will have a great impact on embryo transfer and micromanipulation.

Future prospects for microsurgery in cattle based on results achieved in laboratory animals

Parthenogenesis (production of homozygous diploid mammals)

Parthenogenesis is the development of a gamete without the chromosomal participation of the other sex. There are some natural parthenogenons, amongst reptiles and insects (Rottmann, 1982). Artificial parthenogenesis may be induced by many factors (Beatty, 1957). One successful way is the suppression of the second polar body by cold shock. This procedure works tolerably well in fish resulting in an animal which is self-fertilized. Damaging or removing one of the pronuclei leads to a haploid egg which can be made diploid by cytochalasin. Hoppe and Illmensee (1977) succeeded in producing homozygous diploid uniparental mice by this method (Figure 8).

Cloning

Cloning means, amongst other things, the development of an enucleated egg after transplantation of a somatic cell nucleus. The transplantation of a somatic cell nucleus has not succeeded in mammals but was successful in fish and amphibians. However, the transfer of an embryonic cell nucleus, has succeeded in mice. After isolation and dissociation of the inner cell mass, embryonic nuclei were transplanted into fertilized eggs after removing the pronuclei.

The experimental scheme used for nuclear transplantation in mice by Illmensee & Hoppe (1981) is shown in Figure 9. Blastocysts were collected from donor females on day four (p.c.) and kept in a culture medium. After surgical removal of the zona pellucida, the blastocysts were dissected manually into the inner cell mass (ICM) and trophectoderm (TE), both were then dissociated enzymatically into single cells. An ICM or TE cell was mechanically disrupted by sucking it into a small glass pipette and the cell nucleus, with surrounding cytoplasm, was subsequently injected into a fertilized egg. Following nuclear injection, the genome of the recipient egg was removed by sucking the male and female pronucleus into the micropipette. The nuclear transplant embryos were cultured *in vitro* to the blastocyst stage and then transferred together with some control embryos into the uterus of a pseudopregnant female in order to allow fullterm development. Live-born mice were analyzed chromosomally and biochemically for the genetic markers of the transplanted nuclei. In breeding tests, the nuclear transplant mice were examined for the functional germline transmission of the nuclear transplant genome (Illmensee & Hoppe, 1981).

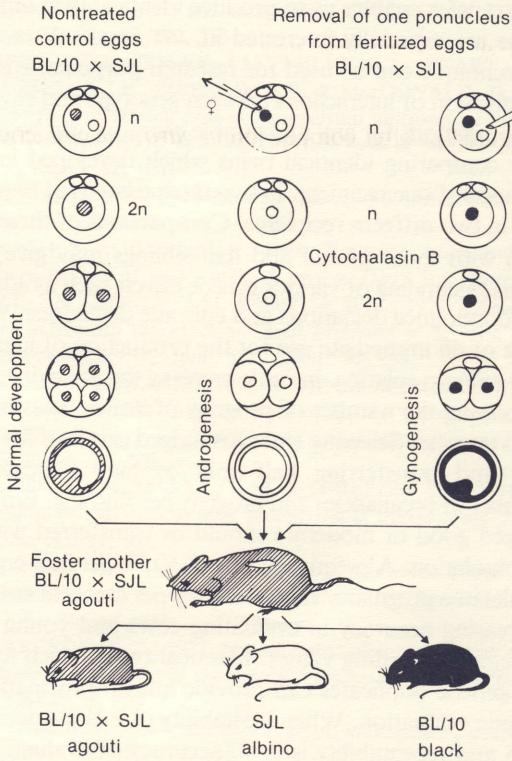

Figure 8 Experimental scheme for the production of homozygous-diploid (isogeneic) mice (Hoppe & Illmensee, 1977.)

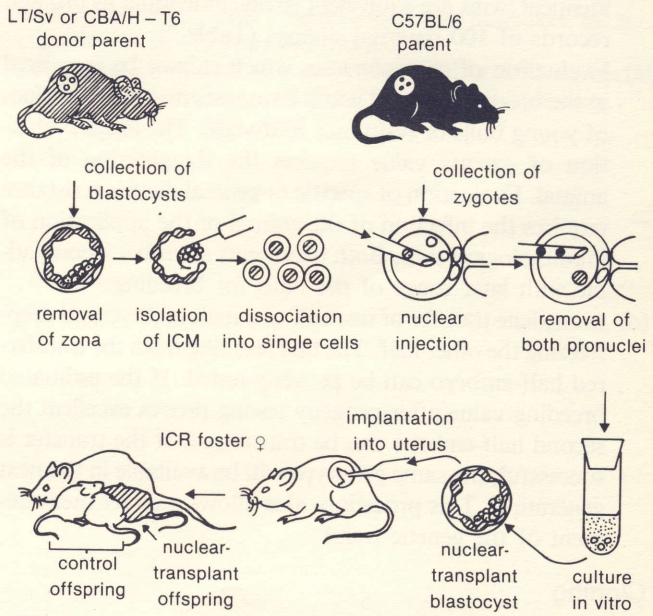

Figure 9 Experimental scheme for nuclear transplantation in the mouse (Illmensee & Hoppe, 1981).

Gene transfer

Selected sequences of DNA, generally including a eucaryotic gene and some bacterial plasmid, have been injected into the pronucleus of the fertilized egg. Both frogs and mice developing from these eggs have been reported to contain the injected sequences. The approach of directly injecting genes into eggs currently offers a promising technique for selectively altering the genetic make-up of an animal.

Potential genetic impact of identical twins, cloning selfing and gene transfer in cattle breeding

Identical twins

Each clone is genetically an exact duplicate occuring naturally only in identical twins. Splitting of embryos and subsequent embryo transfer enables us to produce identical twin animals which we are especially interested in.

The technique can be used for research purposes, *e.g.* for the investigation of interactions between genotype and environment. Correlations for any measurable character can be calculated by comparing identical twins which developed in one uterine horn of one recipient, in two uterine horns of one recipient or in two diffeent recipients. Comparisons of these correlations with those of full and half-siblings may give us a deeper understanding of various genetic effects such as additive effects, dominance deviations and epistatic deviations. When we think of an immediate use for the production of identical twins by embryo-splitting in cattle breeding the following arise:

(a) Increasing the number of progeny of donor cows in embryo transfer. Selecting embryos judged excellent for splitting and transferring half-embryos may increase the number of pregnancies and progeny per flushing. Embryos judged good or moderate should be transferred without manipulation. A pregnancy rate of 60 % per half-embryo results in a pregnancy rate of 120 % per excellent embryo.

(b) Increasing accuracy in evaluating cows and young bulls as regards breeding values. Identical twins which are exact genetic duplicates can provide added information in genetic evaluation. When heritability of a characteristic is 0,25 and repeatability is 0,50, accuracy of evaluation increases from 0,5 (one record per individual) to 0,63 (one record per individual and its identical twin) (Van Vleck, 1981). This example shows that the records per pair of identical twins are equivalent to one individual record and records of 100 paternal siblings (Table 2).

(c) Evaluation of characteristics which cannot be measured in the breeding animal itself. Examples are carcass values of young bulls or resistance to disease. The exact evaluation of carcass value requires the slaughtering of the animal. Evaluation of specific or general disease resistance requires the infection of the animal or the application of immunological tests. Both treatments are often incompatible with later usage of the bulls for breeding.

(d) Immediate transfer of one half of a male embryo and deep-freezing the other half. The bull resulting from the transferred half-embryo can be progeny-tested. If the estimated breeding value after progeny testing proves excellent the second half-embryo can be transferred. If the transfer is successful, the same genotype will be available in the next generation. This procedure also allows a direct measurement of the genetic trend.

Cloning

Identical twins are a clone consisting of only two animals. The technique of cloning aims at creating clones consisting of greater numbers of animals. The application of cloning to animal breeding is therefore similar to the use of identical twins.

Accuracy of evaluation

According to Van Vleck (1981) the accuracy of evaluation of single records on m clones becomes:

$$r = \frac{mh^2}{1 + (m-1)h^2}$$

Table 2 shows that paternal half-siblings do not give nearly as much information about the cow as do her duplicates. Although the accuracy is increased with cloning, the selection intensity factor would be decreased since fewer genetically distinct groups would be available for selection. An optimal balance would have to be found which may or may not be more effective than current selection procedures.

Table 2 Accuracy of evaluation of a cow using records from her clones and her paternal siblings (Van Vleck, 1981)
Assumptions: $h^2 = 0,25$; $w = 0,50$

	Records			
	0	1	2	3
Number of clones				
0	0,00	0,50	0,58	0,61
1	0,50	0,63	0,67	0,69
2	0,63	0,71	0,73	0,75
3	0,71	0,75	0,77	0,78
4	0,75	0,79	0,80	0,81
5	0,79	0,82	0,83	0,83
Number of paternal siblings				
0	0,00	0,50	0,58	0,61
5	0,25	0,53	0,60	0,63
10	0,32	0,55	0,62	0,64
20	0,38	0,58	0,63	0,66
50	0,44	0,60	0,65	0,68
100	0,47	0,62	0,66	0,68

Intensity of selection

The potential usefulness of cloning appears to rely on finding outstanding cows and then mass-producing them. Before mass production careful testing of cows seems necessary. Thus, time required for testing, costs of testing and the probability of finding genetically outstanding cows in a population are crucial points in a breeding programme using cloning. An additional danger is that genetic diversity could be substantially reduced if only a very few cows provided all the clones. There would be a danger of producing cows which could not adapt to changing environmental conditions. Genetically different reserve lines become necessary to maintain the adaptability for future requirements. The history of hybrid seed corn, which gives much the same end result as cloning, provides examples of the potential difficulties of having only limited genetic variability. Another important factor is costs. But it seems too early to speculate about the costs of cloning before we have a technique which can be applied to animal breeding.

Selfing

The assumption is that females can be produced which com-

bine two random X-bearing gametes of a male. Such a procedure would be equivalent to selfing in plants. The apparent advantage would be that the genetic material of the bull could be tested for production characteristics without the usual 50 % dilution by the dam's genes. Females produced by selfing are 50 % inbred. The consequence is that any simple inherited deleterious characteristic would show up and bulls carrying such genes could be culled. The chance that any bulls exist which do not carry deleterious recessive genes is probably quite low. The other problem is that non-lethal effects of inbreeding complicate genetic evaluation, since there are no correction factors available for the effects of inbreeding. The theoretical increase in accuracy, if inbreeding could be discounted, would be that 12 records of 'selfs' would achieve the same accuracy as 50 records of half-sibling daughters (Van Vleck, 1981). In summing up, selfing may be useful in biological research and in testing for lethals. There seems to be no usefulness in selecting for production.

Gene transfer

Practical uses of gene injection could involve the introduction of a desirable gene into large farm animals either to:
(a) influence the animal in a beneficial way; or
(b) cause the animal to manufacture a valuable product.
An example of the first use would be the addition of a growth hormone gene, under the control of the metallothionein promoter, into the gene-pool of livestock in order to increase the efficiency of growth or milk production. Palmiter, Brinster, Hammer, Trumbauer, Rosenfeld, Birnbaum & Evans (1982) have demonstrated that this gene construction does result in elevated growth-hormone in the blood and a dramatic increase in growth-rate in mice.

Another example of the first use is to increase the rate of synthesis of certain proteins. In some cases, an important constituent of the commercial product is either a single protein or a group of related proteins, such as the three casein genes in milk, or the 50 or more keratin genes in sheep. Judging from the recently published results of injection into fertilized eggs, it might not be too difficult to increase the number of genes coded for a particular protein or type of protein and perhaps to increase its rate of synthesis (Robertson, 1982).

An example of the second use would be to introduce a gene for a commercially or medically useful protein into a large animal (Brinster & Palmiter, 1982). The advantage of using animals rather than bacteria or yeast is the ability of the animal to process the product correctly. The biological functions of some proteins in the body are dependent on intracellular processing such as; proteolytic cleavage and glycosylation of hormones or gamma carboxylation of certain glutamic residues of blood clotting factors. Thus, animals could be used in a type of 'gene farming' to produce valuable proteins which could be harvested. According to Brinster & Palmiter (1982), the production of bloodclotting factors to combat haemophilia is a potential use for gene farming.

The main obstacles to the practical application of gene injection seem to be identifing useful DNA sequences and finding suitable promoter – regulator genes. The lack of knowledge of the genome of our farm animals makes it difficult to conceive of a practical application of this technique in the near future.

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