Adsorption of DNA to the sperm of rams, bulls and fallow bucks

A.J. PETERSON, K.M. SCHOFIELD AND R.R. McLAUGHLIN

Ruakura Agricultural Centre, Ministry of Agriculture and Fisheries, Hamilton

ABSTRACT

As a pre-requiste to examine the possibility of using spermatozoa as a vector for the incorporation of foreign DNA into livestock, the ability of sperm from sheep, fallow deer and cattle to bind exogenous DNA was examined.

The DNA was obtained from the plasmid pMK1 linearised with EcORI XhoI and end-labelled with ^{32}P. Fresh sperm from all species bound DNA with maximum binding occurring after 1 hr of incubation. Frozen sperm bound less DNA and formaldehyde treated sperm did not bind. Seminal plasma inhibited binding as did heparin and also high divalent cation concentrations. Capacitated ram sperm bound more than uncapacitated (64% vs 40%) and uterine fluids from sheep two-days post-estrus did not interfere with DNA binding to the capacitated ram sperm.

Increasing the concentration of washed capacitated ram sperm from 1 x 10^6/ml to 30 x 10^6/ml and with a DNA concentration of 0.25 mg/ml decreased the number of molecules of DNA bound from 38,000 to 1,600. There was wide variation among rams in the percentage of DNA bound after 1 hr incubation, ranging from 15-80% (n=7).

These results suggest that this procedure may be a possible method for the incorporation of foreign DNA into livestock by intrauterine insemination.

INTRODUCTION

The most widely used technique for the production of transgenic animals requires the use of labour-intensive microinjection of foreign DNA into the pronuclei of individual fertilised eggs; a technique that requires expensive, specialised equipment. In addition, in the case of sheep and cattle, visualisation of the pronuclei is difficult and confined to only a few critical hours after conception.

A recent report described the production of transgenic mice using spermatozoa as vectors for introducing foreign DNA in eggs at fertilisation (Lavitrano *et al.*, 1989). Using an *in vitro* fertilisation (IVF) method with subsequent implantation into surrogate mothers a success of 30% was reported with a potential of generating some five times more transgenic offspring than with the micro-injection method.

Two other reports suggest that the DNA adsorption procedure may produce transgenic animals. Brackett *et al.*, (1971) presented the first, although indirect, evidence of sperm mediated transfer of viral DNA into rabbit embryos after intra-uterine artificial insemination. More recently, Arezzo (1989) reported that transgenic sea urchin blastula can be produced if the eggs are fertilized with sperm incubated with foreign DNA. In neither report however, was any evidence of chromosomal integration of the foreign DNA given.

The present experiments were performed to examine the dynamics of DNA binding to the spermatozoa of sheep, cattle and deer. This was a pre-requisite to examine the possibility of using spermatozoa as a vector for the production of transgenic livestock in conjunction with intra-uterine artificial insemination.

EXPERIMENTAL

DNA The DNA used was obtained from the plasmid pMK'. This consists of the mouse metallothionine-1 gene fused to the coding sequence of the thymidine kinase gene isolated from the herpes simplex virus, type 1. This construct was inserted between the EcORI and PvuII sites of pBX322, a high copy number variant of pBR322 (Stuart *et al.*, 1984). The plasmid was linearised with EcORI and XhoI and end-labelled with ^{32}P (Sambrook *et al.*, 1989).

Spermatozoa

Fresh ejaculated ram and fallow deer semen was obtained

from the AB Centre at Ruakura. Sperm density was determined by spectro-photometry immediately after collection. Samples were transported in sealed centrifugation tubes in a thermus container at 37°C. Fresh bull semen was obtained from the NZ Dairy Board AB Centre, Newstead, N.Z..

Incubations

The fresh semen samples were prepared in phosphate-buffered saline supplemented with 0.3% bovine serum albumin (PBS, pH 7.0). Calcium ion concentrations were modified by the addition of $CaCl_2$ to a final concentration of either 1 or 10 mM.

Initial experiments indicated the presence of seminal plasma in the incubates caused a marked reduction in the amount of ^{32}P bound to the spermatozoa. Accordingly, individual ejaculates were suspended in 10 ml PBS, mixed gently and centrifuged at 200 g for 10 minutes. The supernatents were aspirated and the procedure repeated. The final sperm pellet was gently resuspended in 1 ml PBS then diluted into the required volume of PBS to give a final concentration of 2 x 10^6/ml.

In experiments to assess the effect of capacitation on binding of the foreign DNA, individual ejaculates were left for 0, 1, or 2 hours at 37°C before washing and diluting. The effects of adding increasing amounts of the polyanionic competitor, heparin to the incubation media was measured by adding increasing concentrations of heparin from 0.004 to 40 mg/ml either simultaneously with washed capacitated ram sperm and labelled DNA or 30 minutes after the mixing of the sperm and DNA.

In experiments to determine if the foreign DNA was incorporated into the sperm or bound to the membrane surface, washed capacitated fallow deer sperm was incubated with labelled DNA for 30 minutes before the addition of the enzyme DNAase (20 ml of a 0.5 mg/ml solution in PBS; Promega, Wisconsin, U.S.A.). Binding was assessed for a further 60 minutes. These experiments contained an extra set of control incubates with the same magnesium ion concentrations as those in the DNAase incubates (10 mM).

To assess whether uterine fluids would affect the binding of DNA to sperm, uterine flushings from 20 ewes collected surgically on days 1 and 2 after oestrus were combined, centrifuged and the supernatent freeze-dried. The lyophilised material was reconstituted in PBS to 1/10 of its original volume. This was then incubated simultaneously with labelled DNA and capacitated and washed ram sperm for 90 min.

The effects of incubating sperm and DNA in the presence of high protein and calcium ion concentrations such as used in current ovine *in vitro* fertilisation protocols were determined by supplementing the incubation media with 20% sheep serum and either 1 or 10 mM calcium.

The variation in the ability of sperm from individual rams to bind labelled DNA was assessed in seven individuals. Fresh, capacitated and washed sperm was incubated with labelled DNA and the amount of DNA bound after 60 min measured.

Determination of bound DNA

The ^{32}P-labelled DNA (0.25 mg/ml) and sperm (2 x 106/ml) were mixed and incubated at 37°C on a shaker. At 5, 10, 15, 30, 60 and 90 min intervals 1 ml was aliquoted in duplicate and centrifuged at 200 g for 5 min. The supernatants were aspirated and the sperm pellet resuspended in 1 ml PBS, recentrifuged for 5 min as above and the supernatant removed. This process was repeated twice. The washed sperm pellets were solubilised overnight with 0.4 ml NCS solution (Amersham, England) and then transferred with rinsing into scintillation vials and the radioactivity measured in b-Scintillation counter.

In all experiments, control tubes containing only labelled DNA and buffer were run in duplicate.

RESULTS

Allowing ram semen to stand for 2 hr before washing and incubation caused significantly more DNA to be subsequently bound to the sperm (Table 1). This observation was confirmed with fallow deer and bull sperm.

TABLE 1 % DNA (± s.e., n=4) bound to sperm left for 0, 1 or 2 hrs before dilution and washing. Sperm and DNA concentration 30 x 10^6/ml and 0.25 mg/ml respectively.

	Time (minutes)			
	5	15	30	60
0 hr	10 (3)	20 (6)	25 (6)	27.5 (6)
1 hr	15 (5)	35 (6)	45 (6)	51 (8)
2 hr	28 (6)	45 (8)	56 (5)	63 (6)

TABLE 3 % DNA bound in the presence of high protein and Ca++ concentrations

	Time (minutes)		
Treatment	5	30	60
Sperm + 1mM Ca ++	2.4	4.8	4.2
Sperm + 10 mM Ca ++	2.1	21	19.5
10 mM Ca ++	4.8	21.9	17.5

TABLE 2 Displacement of labelled DNA from ram sperm by (a) increasing concentrations of heparin (T=0; DNA and heparin added simultaneously; T=30; sperm and DNA incubated for 30 minutes before addition of heparin) and (b) by the addition of DNAase and the presence of 10 mM mg++ in fallow buck sperm.

(a)				Heparin Concentration (mg/ml)			
% DNA bound			0.004	0.04	0.4	4	40
T=0			100	100	44.2	8.2	4.1
T=30			100	100	85.8	22.1	13.1
(b)				Time From DNAase (min)			
	-25	-20	-15	0	+10	+20	+30
Control	11	15	18	25	29	31	34
+DNAase				26	1	0.5	0.2
+ 10mM++				28	5	3	4

Adding increasing amounts of heparin to the incubation media progressively displaced more labelled DNA from the sperm. If, however, sperm and the DNA were incubated for 30 minutes before the addition of DNA, more DNA remained subsequently bound (Table 2a). Within 10 min of adding DNAase to fallow deer sperm incubates almost all of the labelled DNA is removed from the sperm. In these experiments DNA was even removed in the presence of 10 mM magnesium in the buffer (Table 2b).

Incubating labelled DNA and sperm for 90 min in the presence of sheep uterine fluids had no effect on the binding of the DNA to the sperm; 14.2 ± 2.6% being bound in the absence and 12.9 ± 3.1% (±SD, n=4) in the presence of uterine fluids.

Incubating sperm and DNA using high protein and calcium ion concentrations had a deleterious effect on the binding of DNA to the sperm (Table 3). These high concentrations of calcium precipitated DNA even in the absence of sperm.

There was a wide animal variation in the amount of DNA bound ranging from 17 to 80% after 60 minutes of incubation (Table 4).

TABLE 4 Variation among rams in % DNA bound after 60 min incubation

			Ram			
1	2	3	4	5	6	7
17.4	40.3	42.7	62	65	68.6	77.7

DISCUSSION

The present experiments indicate that foreign DNA does bind to capacitated and washed sperm from rams, bulls and fallow bucks. Uterine fluids do not appear to affect the binding of foreign DNA but the binding is

markedly reduced in the presence of heparin and high protein and calcium concentrations. Although the uterine fluids of sheep do contain proteins, glycosaminoglycans and calcium (Lee *et al.*, 1986) the concentrations would appear too low to interfer with the sperm-DNA binding once the DNA has been adsorbed. Using DNA-adsorbed sperm to generate transgenic livestock is, therefore, incompatible with current *in vitro* fertilisation/maturation procedures used to produce sheep and cattle embryos. However, the method could be assessed using intra-uterine artificial insemination. The wide among animal variation in the ability to bind DNA suggests that before this procedure can be tested to generate transgenic livestock,carrier spermatozoa must be screened before use.

The data does not indicate if DNA is preferentially bound to specific regions of the sperm. Lavitrano *et al.*, (1989) stated that in mice most of the foreign DNA is localised in a specific portion of the sperm head and that extensive washing with dextran sulphate removed only about 50% of the foreign DNA suggesting a stable incorporation. Bracket *et al.*, (1971) showed that 30-35% of rabbit sperm were capable of incorporating ^{3}H-labelled DNA on the postacrosomal cap region of the sperm. Interestingly, Handrow *et al.*, (1984), using a fluoresence label, have shown, that heparin binds preferentially to the tail and midpiece of bull, monkey and rabbit sperm. Since heparin, (more strongly polyanionic than dextran sulphate), high magnesium and calcium concentrations and DNAase do competitively remove DNA from ram and fallow deer sperm, the foreign DNA would appear to be membrane bound in these species and not as suggested by Lavitrano *et al.*, (1989) for mice, to have penetrated into the sperm head. Table 2 however, does suggest that foreign DNA may, under certain conditions be stably associated with ram sperm.

Although intensive work world wide using mice has failed to repeat the original observations of Lavitrano *et al.*, (1989) using both *in vitro* fertilisation (Brinster *et al.*, 1989) and artificial insemination (Al-Shawi *et al.*, 1990), a recent report indicates that transgenic pigs have been produced by intra-uterine artificial inseminations using DNA-adsorbed spermatozoa (Gandolfi *et al.*, 1990). Thus further work into the production of transgenic livestock using spermatozoa as vectors may be warranted.

REFERENCES

Al-Shawi, R.; Ansell, J.D.; Bishop, J.O.; Simons, J.P.; West, J.D. (1990). Failure to produce transgenic mice by exposing spermatozoa to DNA. *Mouse Newsletter*. *98*: 224.

Arezzo, F. (1989). Sea urchin sperm as a vector of foreign genetic information. *Cell Biology International Reports 13*: 391-404.

Brackett, B.G.; Baranska, W.; Sawicki, W.; Koprowski, H. (1971). Uptake of heterologous genome by mammalian DNA and its transfer to ova through fertilisation. *Proceedings of the National Acadamy of Sciences 68*: 353-357.

Brinster, R.L.; Sandgren, E.P.; Behringer, R.R.; Palmiter, R.D. (1989). No simple solution for making transgenic mice. *Cell 59*: 239-241.

Gandolfi, F.; Lavitrano, M.; Camioni, A.; Spadafora, C.; Siracusa, G.; Lauria, A. (1990). The use of sperm-mediated gene transfer for the generation of transgenic pigs. *Journal of Reproduction and Fertility*. Abstract Series *4*: 10.

Handrow, R.R.; Boehm, S.K.; Lenz, R.W.; Robinson, J.A.; Ax, R.L. (1984). Specific binding of the glycosaminoglycan 3H-Heparin to bull, monkey and rabbit spermatozoa *in vitro*. *Journal of Andrology 5*: 51-63.

Lavitrano, M.; Camioni, A.; Fazzio, V.M.; Dolci, S.; Farace, M.G.; Spadafora, C. (1989). Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. *Cell 57*: 717-723.

Lee, C.N.; Clayton, M.K.; Bushmeyer, S.M.; First, N.L.; Ax, R.L. (1986). Glycosaminoglycaus in ewe reproductive tracts and their influence on acrosome reactions in bovine spermatozoa *in vitro*. *Journal of Animal Science 63*: 861-867.

Sambrook, J.; Fritsch, E.F.; Maniatis, T. (eds.) 1989. *Molecular Cloning, A Laboratory Mannual, 2nd* Ed. Cold Spring Harbour Laboratory Press.

Stuart, G.W.; Searle, P.F.; Chen, H.Y.; Brinster, R.L.; Palmiter, R.D. (1984). A 12-base DNA motif that is repeated several times in metallothioein gene promoters confers initial regulation to a heterologous gene. *Proceedings of the National Acadamy of Science (U.S.A.) 81*: 7318-7322.