The fate of DNA injected into mammalian oocytes and zygotes at different stages of the cell cycle

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Summary. The stability of exogenous DNA microinjected into the cytoplasm at different stages of the meiotic cycle and after pronuclear formation was examined in ungulate species. Metabolism of the injected 1201 base pair (bp) DNA was examined by Southern blotting. Similar levels of metabolism of the injected DNA were detected in pig, sheep and bovine oocytes before germinal vesicle breakdown, in which about 30–40% of detected DNA was ligated into higher-molecular-weight forms. Porcine metaphase oocytes and pronuclear zygotes showed a reduced ability to ligate the exogenous DNA. In contrast, sheep and bovine metaphase oocytes and zygotes showed increased levels of ligation and, at the pronuclear stage, generated significant amounts of extremely large (> 15 kbp) ligation products. These results are discussed in the context of maternal precursors and metabolic activities in the egg.

Keywords: oocyte; zygote; microinjection; DNA stability; meiotic cycle; pig; sheep; cow

Introduction

In higher organisms, the oocyte provides the precursors required for early embryonic development. In *Xenopus*, a large store of nucleic acid precursors, enzymes and RNAs is established to support rapid cell division (12 cell cycles in less than 12 h; for review see Laskey, 1985). In comparison, early mammalian development is much slower; for example, mouse and ungulate zygotes take about 24 h to complete the first cell cycle and transcribe embryonic genes before five cell cycles are completed (Bensaude *et al.*, 1981; Telford *et al.*, 1990).

One way in which the availability of some of these oocyte precursors can be assessed is by following the fate of exogenous DNA injected into the oocyte and fertilized egg. In certain cases, foreign DNA is apparently assembled into native chromatin. Briefly, it was found that single-stranded DNA and linear DNA are degraded in the nucleus and cytoplasm of the *Xenopus* oocytes. Covalently closed DNA, although degraded in the cytoplasm, is stable in the nucleus of oocytes (Wyllie *et al.*, 1977, 1978). However, the cytoplasm of the mature egg appears unable to degrade microinjected DNA but ligates circular DNA (Laskey *et al.*, 1977), and constructs a 'mininucleus' around foreign DNA – especially large (> 40 kbp) DNA (Forbes *et al.*, 1983). In these structures, the mininuclei contain nuclear lamin proteins and possess a lipid bilayer membrane (Forbes *et al.*, 1983; Trendelenburg *et al.*, 1986). Circular DNA is also a more efficient template for transcription (Harland *et al.*, 1983). Studies on the stability of DNA injected into *Xenopus* eggs after fertilization indicate that injected linear molecules can persist to early embryonic stages when they appear to become associated with high-molecular-weight DNA. Furthermore, it appears that in approximately 60% of cases linear DNA can integrate into the *Xenopus* genome although covalently closed circular DNA only integrated in 10–30% of cases (Etkin *et al.*, 1984).

Fewer studies have been reported in mammals. Brinster *et al.* (1985) have shown that foreign DNA injected into the cytoplasm of the pronuclear zygote is not readily incorporated into the host

genome. However, DNA microinjected into the cytoplasm of tissue culture cells is efficiently integrated and suffers only minimal mutation in the host cell (Kopchick & Stacey, 1984). The development of *in vitro* culture techniques for mammalian oocytes (Staigmiller & Moor, 1984), has enabled the relative stability of DNA during oocyte maturation and after fertilization of the mature egg to be monitored. A DNA probe containing multiple repeat sequences was prepared for our study and used to assess DNA stability in interphase and meiotic cytoplasm and in the cytoplasm of pronuclear zygotes. The results show species differences in the stability of exogenous DNA. In sheep and bovine cells, metabolizing activities appear to increase during oocyte maturation and after fertilization. In pigs, however, DNA metabolism is decreased in the cytoplasm of the metaphase oocyte and zygote.

Materials and Methods

Plasmid DNAs

Clone p16 was supplied by Dr Wu (Cornell University, Ithaca; Yang *et al.*, 1983). The *Hind*III–*Eco*RI fragments (Fig. 1a) were subcloned into the vector pGEM4 (Promega Biotec) using standard techniques (Sambrook *et al.*, 1989). The 1201 bp *Eco*RI insert of p16 was prepared by electroeluting the fragment from agarose gels, purified by passage over Sephadex G-50 and redissolved at 100–500 ng μl^{-1} in $\text{TE}_{0.1}$ (10 mmol Tris-HCl l^{-1} (pH 7.5), 0.1 mmol EDTA l^{-1}). Control experiments with genomic DNA from a variety of species were used to determine whether p16-related sequences were present in the genomes of ungulate species.

Fig. 1. p16-derived DNA probes. (a) Construction of DNA probes. The 1201 bp insert of p16 shown in the upper panel, consists in part of two repetitive elements – a 66 bp sequence present in eight tandem copies (cross-hatched boxes) and a single copy of a 300 bp *Alu*-type sequence (striped box; Yang *et al.*, 1983). The *Eco*RI fragment containing the 66 bp repetitive sequences was subcloned into the vector pGEM4 (lower panel). [^{32}P]-labelled RNA was produced by linearizing the resultant plasmid, pGp16A, at the *Hind*III site and transcribing *in vitro* using SP6 RNA polymerase (Melton *et al.*, 1984). (b) Hybridization of [^{32}P]-labelled pGp16A riboprobes to *Eco*RI-cleaved genomic DNAs. Genomic DNA, isolated from male livers was cut with *Eco*RI, electrophoresed on a 0.6% agarose gel (left panel) and transferred by nylon membrane. The blot was hybridized with [^{32}P]-labelled pGp16A riboprobes (right panel). Only the human DNA (lane 6) hybridized to any significant extent to pGp16A. Lane 1, mouse DNA; lane 2, rat DNA; lane 3, sheep DNA; lane 4, bull DNA; lane 5, pig DNA; lane 6, human DNA.

Preparation and injection of oocytes

Oocytes were obtained from nonatretic follicles dissected from the ovaries of sheep, pigs and cows at slaughter. Before microinjection, germinal vesicle stage oocytes were recovered from follicles and all but the last few layers of

follicular cells were removed using graded micropipettes as described by Osborn & Moor (1982). Metaphase II oocytes were obtained after *in vitro* maturation (Staigmiller & Moor, 1984) which lasted 24 h for the sheep and cow and 44 h for the pig. Before microinjection, the expanded cumulus was removed by a short treatment (1–3 min) with hyaluronidase (final concentration 0.1 mg ml^{-1}), followed by micropipetting. Pronuclear eggs were injected 18 h after *in vitro* fertilization of oocytes matured in culture (sheep: Crozet *et al.*, 1987; cow: Parrish *et al.*, 1986; pig: Yoshida *et al.*, 1990). In all cases the success of fertilization was assessed by cleavage (for groups analysed $\geq 20 \text{ h}$ after fertilization) or by the morphological analysis of 10% of each group of eggs (for those analysed 3 h after fertilization). During microinjection, oocytes and eggs were held in tissue culture medium (TCM) 199 containing $25 \text{ mmol Hepes l}^{-1}$ supplemented with 10% calf serum, sodium pyruvate (0.4 mmol l^{-1}) and kanamycin sulphate ($50 \mu\text{g ml}^{-1}$). Up to 10 pl of DNA solution was injected using a calibrated micropipette (Moor & Powell, 1989). In control experiments using TCM 199, phosphate-buffered saline (PBS: Paul, 1970) and $\text{TE}_{0.1}$ as carrier solutions, we detected no differences in survival, morphology or timing of developmental changes (R. M. Moor, unpublished observations).

Oocytes and fertilized eggs were cultured after DNA injection for between 3 and 30 h at 38.5°C in 5% CO_2 with saturated humidity in TCM 199 containing $25 \text{ mmol sodium bicarbonate l}^{-1}$ and supplemented with 10% fetal calf serum, sodium pyruvate (0.4 mmol l^{-1}) and kanamycin sulphate ($50 \mu\text{g ml}^{-1}$). At harvest, groups of 15–30 injected oocytes or eggs were quick-frozen in liquid nitrogen, before storage at -80°C .

DNA preparation

DNA was extracted from male livers of mouse, rat, bull, ram and boar and from human sperm. The freshly isolated livers were coarsely chopped and homogenized for 30 s in PBS using a Polytron homogenizer at a setting of 4. One-tenth volume of $3 \text{ mol sodium acetate l}^{-1}$, $0.2 \text{ mol Tris-HCl l}^{-1}$ (pH 7.5) $0.1 \text{ mol EDTA l}^{-1}$, $4 \text{ mg proteinase K ml}^{-1}$ was added to the cell suspensions and mixed well. Sodium dodecyl sulphate was added to a final concentration of 0.5%, mixed well, and the cell lysates incubated at 56°C for 4 h. DNA was prepared by phenol:chloroform (1:1) extraction, followed by chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). Between 3 and $6 \mu\text{g}$ of each DNA were cleaved with *EcoRI* under standard conditions (Sambrook *et al.*, 1989), electrophoresed on a 0.6% agarose gel and transferred to a nylon membrane according to the protocol of Church & Gilbert (1984).

DNA was extracted from batches of oocytes and embryos as follows. The oocytes (in $10\text{--}20 \mu\text{l}$ of PBS) were melted into $100 \mu\text{l}$ of lysis buffer ($0.3 \text{ mol sodium acetate l}^{-1}$, $20 \text{ mmol Tris-HCl l}^{-1}$ (pH 7.5), $10 \text{ mmol EDTA l}^{-1}$, 0.5% SDS, $100 \mu\text{g proteinase K ml}^{-1}$, $5 \mu\text{g salmon sperm DNA ml}^{-1}$) and incubated at 56°C for 30 min. DNA was purified by two extractions with phenol:chloroform (1:1), two extractions with chloroform and one extraction with ether and then precipitated with 2 volumes of ethanol and collected by centrifugation at $10\,000 \text{ g}$ for 5 min. The DNA pellets were dissolved in $8 \mu\text{l}$ of water, mixed with sample buffer and applied to 0.6% agarose mini gels ($75 \text{ mm} \times 50 \text{ mm}$). After electrophoresis at 50 V for 2 h the DNA was transferred to nylon membrane by alkaline blotting. For subsequent studies, only those samples that gave quantitative recovery of the carrier salmon sperm DNA were used.

Hybridization

Labelled riboprobes were prepared according to the manufacturer's instructions (Promega Biotec; Melton *et al.*, 1984). The reactions contained: $14 \text{ mmol Tris-HCl l}^{-1}$ (pH 7.5); $6 \text{ mmol MgCl}_2 \text{ l}^{-1}$; $2 \text{ mmol spermidine l}^{-1}$; $10 \text{ mmol sodium chloride l}^{-1}$; $10 \text{ mmol dithiothreitol l}^{-1}$; 1 unit pancreatic ribonuclease inhibitor (RNasin) μl^{-1} ; 0.5 mmol l^{-1} of ATP, CTP and GTP; $12 \mu\text{mol UTP l}^{-1}$; $1 \mu\text{g linearized template pGp16A DNA}$, $50 \mu\text{Ci } [^{32}\text{P}]\text{UTP}$ ($1000 \text{ Ci mmol}^{-1}$) and 10 U SP6 polymerase. Reaction vessels were incubated at 37°C for 45 min. Input DNA was removed by DNAase digestion and the RNA probe purified by phenol extraction and repeated ethanol precipitation from $2.5 \text{ mol ammonium acetate l}^{-1}$ and 2 volumes of ethanol.

Filters were prehybridized in $0.5 \text{ mol sodium phosphate l}^{-1}$ (pH 7.2), 7% SDS at 65°C for 1 h (Church & Gilbert, 1984). Hybridization was in the same buffer plus $1.0 \times 10^6 \text{ dpm of probe ml}^{-1}$ (previously denatured by heating in 50% formamide at 65°C). Hybridization was at 65°C for 16 h. Filters were washed once for 5 min in $40 \text{ mmol sodium phosphate l}^{-1}$ (pH 7.2), 0.1% SDS at room temperature and then twice for 30 min in $40 \text{ mmol sodium phosphate l}^{-1}$, (pH 7.2), 1% SDS at 65°C . Autoradiographs were exposed at -70°C for 1–10 days and then scanned using a Joyce–Loebl Chromoscan. Peak areas were estimated for each size class of DNA (1.2, 1.5–15 and $> 15 \text{ kbp}$), and these areas were then normalized, arbitrarily setting the normalized area of the germinal vesicle 1.2 kbp band to 1. The sensitivity of detection was estimated using a reconstruction experiment in which the equivalent of p16 insert DNA in 0.1, 1, 10, 20, 100 and 200 oocytes (i.e. 0.3–600 pg) was extracted for electrophoresis and then hybridized.

Results

Preparation of DNA constructs

To provide an efficient marker for injected DNA, a repetitive sequence not found in ungulate genomes was used. This was a plasmid containing a series of eight tandem copies of the 66 bp repeat

sequence (p16, Fig. 1a); this DNA does not hybridize with genomic DNA of the ungulate species (Fig. 1b). In this plasmid (Fig. 1a) the tandem array that lies within a 750 bp *HindIII-EcoRI* fragment is located close to a member of the human *Alu* family. The insert, a 1201 bp *EcoRI* fragment, was excised and used for microinjection. The 750 bp *HindIII-EcoRI* fragment was subcloned and used to generate riboprobes.

The fate of linear DNA in porcine oocytes and zygotes

To investigate the stability of linear DNA, we dissolved the 1201 bp *EcoRI* insert of p16 in TE_{0.1}, at a concentration of 400 µg ml⁻¹ and, using calibrated micropipettes, 7 µl was microinjected into individual cells. The following stages were used: germinal vesicle oocytes, in which the nuclear membrane is intact; metaphase II oocytes, in which the nuclear membrane has broken down and the chromosomes are free in the cytoplasm; and pronuclear zygotes after fertilization, when both pronuclei are present. Batches of 15–25 injected cells were pooled and cultured for 6 or 30 h. At the end of the culture period, DNA was isolated and analysed by Southern blotting. Since there was little difference in cultures of 6 and 30 h (data not shown) representative samples are shown for each stage.

Residual DNA extracted from the needle after microinjection was electrophoresed, and the autoradiograph (Fig. 2, lane 7) shows that it migrated as a single 1201 bp band. Control reconstruction experiments indicated that the sensitivity of detection was equivalent to about 0.1 oocyte per band i.e. ≈ 0.2 pg p16 DNA per band. In germinal vesicle oocytes (Fig. 2, lanes 1 and 2), most of the input DNA remained as a 1201 bp monomer, although ligation products were detected. These ligation products represented a series of multimers of the input 1201 bp by monomers, such as 2.4, 3.6 and 4.8 kbp, and extended to very small amounts of 12 kbp. Other less abundant species were also detected. Metaphase II oocytes gave a pattern of DNA metabolism with even lower levels of ligation, the bulk of the DNA persisting as input, monomeric 1201 bp (Fig. 2, lanes 3 and 4). The products in pronuclear zygote (Fig. 2, lanes 5 and 6) were similar to those obtained in metaphase II oocytes. Representative autoradiographs were analysed using a Joyce–Loebl Chromoscan and the relative amounts of DNA in three size classes – 1.2, 1.5–15 and > 15 kbp – were estimated (Fig. 5a). Later stages showed a reduced ability to ligate the injected DNA. Between 80 and 90% of the input DNA that was detected migrated as monomeric species in metaphase II oocytes and pronuclear zygotes.

The fate of DNA in bovine oocytes and zygotes

Bovine germinal vesicle and metaphase II oocytes and bovine pronuclear zygotes were prepared and microinjected with p16 DNA (Fig. 3). Densitometry of several samples of each stage was carried out and the DNA classified into three categories as for porcine cells (Fig. 5b). The fate of DNA in the bovine germinal vesicle oocytes was similar to that for porcine GV oocytes: $\approx 60\%$ of the input DNA persisted in a monomeric form (Fig. 3, lanes 1 and 2). The remaining DNA formed a ligation series of multimers of the input 1201 bp DNA extending to about 10 kbp. A similar result was also obtained with metaphase II oocytes; 60% monomeric p16 DNA and a ligation series (Fig. 3, lanes 3 and 4). However, a different profile was obtained with pronuclear zygotes in which about 30% of the detected DNA was monomeric, a similar proportion of the detected DNA ranged in size from 1.5 to 15 kbp and about 40% was greater than 15 kbp and comigrated with the largest size marker, at the exclusion limit of the gel. Thus, a greater proportion of detected DNA was ligated into higher-molecular-weight multimers at the pronuclear stage than at the oocyte stages examined (Fig. 5b); note however that lower levels of DNA are recovered at this stage.

The fate of DNA in ovine oocytes and zygotes

Ovine germinal vesicle oocytes, metaphase II oocytes and pronuclear zygotes were microinjected with p16. After culture, the extracted DNAs were analysed by agarose gel electrophoresis,

Fig. 2. Metabolism of p16 DNA in porcine cells. Porcine oocytes or zygotes were microinjected with p16 DNA and cultured *in vitro*. DNA was extracted, electrophoresed on 0.6% agarose gels and transferred to nylon membranes. The membranes were hybridized with [32 P]-labelled pGp16A riboprobes, washed and autoradiographed for 3–6 days. Size markers, derived from parallel lanes of plasmid and phage DNAs, are indicated at the left. Lanes 1 and 2, germinal vesicle oocytes; lanes 3 and 4, metaphase II oocytes; lanes 5 and 6, pronuclear zygotes; lane 7, residual DNA from needle after injection.

blotting and hybridization (Figs 4 and 5c). DNA from injected germinal vesicle oocytes was electrophoresed (Fig. 4, lanes 1 and 2). The majority of detected DNA migrated as a 1201 bp band with some apparent products of ligation that formed a ladder of multimers of the 1201 bp input DNA.

Extensive ligation was detected in the metaphase stage ovine oocytes (Fig. 4, lanes 3 and 4): $\approx 60\%$ of detected DNA was between 1.5 kbp and 15 kbp and $\approx 7\%$ was > 15 kbp (Fig. 5c). At the pronuclear stage, there was extensive ligation of p16 DNA (Fig. 4, lanes 5 and 6). More than 60% of detected DNA migrated as species > 15 kbp, and $< 10\%$ migrated at 1.2 kbp, the size of the injected p16 insert. Thus, ovine oocytes appear capable, at all stages, of considerable modification of input linear DNA.

Discussion

The maturation of the oocyte and the early cleavage divisions in mammalian development provide a unique opportunity to investigate the stability of foreign DNA during phases of the meiotic and mitotic cell cycles. In these studies, we assayed for cytoplasmic enzyme activities that can metabolize DNA, to determine whether there are differences between the cytoplasm of the germinal vesicle oocyte, the metaphase II oocyte and the pronucleate zygote.

Fig. 3. Metabolism of p16 DNA in bovine cells. Bovine oocytes or zygotes were microinjected and DNA analysed. Lanes 1 and 2, germinal vesicle oocytes; lanes 3 and 4, metaphase II oocytes; lanes 5 and 6, pronuclear zygotes. Size markers are indicated to the left.

We examined the fate of foreign DNA of relatively small size after injection into the cytoplasm. Between 0.2 and 2.5 pg of 1201 bp DNA from p16 was introduced into the cells. The diploid content of a mammalian cell is about 6 pg (Britten & Davidson, 1971) and in most experiments less than the equivalent of a haploid genome was injected (about 1.4 pg) of DNA. We would not expect this quantity of DNA to deplete dramatically the cellular resources of proteins that interact with DNA. For example, Clarke & Masui (1987) have shown that mouse oocytes can transform two spermatozoa (i.e. 6 pg of DNA) into metaphase chromosomes. However, the fragment used in our experiments represents a very large number of 'ends' of DNA and thus we might not have expected to detect end-modifications of DNA. In fact, we were able to detect ligation in all species of germinal vesicle oocytes, metaphase oocytes and in pronuclear zygotes, i.e. both in the presence and absence of the nuclear membrane. We do not know if such activities are induced by the presence of large numbers of 'ends' of DNA, but the observation that ligation is detected after about 3 h culture of cells containing the injected DNA suggests that induction, if such occurs, is quite rapid and efficient. Owing to the length of time taken for these injection series, we are currently unable to measure the metabolism during shorter culture periods.

Our experiments show that there are differences in DNA stability, both among the three species at equivalent developmental stages and, in most cases, among the developmental stages in any given species. The cytoplasm of porcine cells was not very effective at the ligation of the 1201 bp p16 DNA; the most significant metabolism was at the germinal vesicle stage, when some 40% of the detected DNA was metabolized to higher-molecular-weight products. In contrast, we detected

Fig. 4. Metabolism of p16 DNA in ovine cells. Ovine oocytes or zygotes were microinjected and DNA analysed. Size markers are indicated to the left. Lanes 1 and 2, germinal vesicle oocytes; lanes 3 and 4, metaphase II oocytes; lanes 5 and 6, pronuclear zygotes.

maximum activities of DNA ligation, in terms of relative amounts of largest product produced, in both ovine and bovine pronuclear zygotes. However, based on our recoveries as determined by peak areas, DNA losses were lowest in porcine zygotes and highest in ovine oocytes.

In general, the germinal vesicle oocytes of all three species metabolized input DNA to similar extents. Thereafter, the three species differ somewhat, and the bovine and ovine oocytes both show considerable metabolism at the metaphase II stage. Paradoxically, this is the stage at which maternal chromosomes are free in the cytoplasm; we assume that oocyte chromosomes are protected from the activities we detect, using p16 DNA, either by complexing with proteins or by the special structure of their termini. In all three species, we recovered similar amounts of DNA at the germinal vesicle and metaphase II stages. Although DNA injected into *Xenopus* oocytes can form a nucleosomal structure, no mininuclei were detected in our studies (C. Galli, unpublished observations). It would be interesting, although technically very demanding, to determine if DNA injected into mammalian cells will form nucleosomal structures comparable to those in *Xenopus*. Surprisingly, we found that the cytoplasm of bovine and ovine zygotes efficiently ligated DNA into very large molecules (Figs 3, 4 and 5) although this ligation occurred against a loss of DNA which may result from degradation. In each case, the most abundant category of DNA was represented by molecules over 15 kbp in length.

Fig. 5. Quantification of DNA detected in ungulate cells. Autoradiographs, exposed within the linear range of the film, were scanned using a Joyce-Loebl Chromoscan and peak areas were calculated. The peak areas were incorporated into the categories; (i) > 15 kbp, (ii) 1.5–15 kbp and (iii) 1–2 kbp, equivalent to input DNA. For each species, these peak areas were normalized against the germinal vesicle 1201 bp band peak area, to reflect differences in detection of DNA. The average peak area for this band was similar in all species. Three or four samples were scanned for each sample. The standard errors of the means are indicated. (a) porcine cells; (b) bovine cells; (c) ovine cells.

The role and significance of these developmental and species differences in DNA metabolism remains unclear. It may be that the cytoplasmic activities in zygotes are required to support DNA replication during post-fertilization development before the activation of the embryonic genome. It is known that the switch to embryonic gene activity occurs at the four- to eight-cell stage in the pig and at the 16- to 32-cell stage in the sheep and cow (Crosby *et al.*, 1988; Telford *et al.*, 1990). The elevated levels of DNA metabolism (both in terms of ligation and degradation) that we find in eggs from sheep and cow may be correlated with the extended period of development that depends on maternally inherited enzyme activities. The increased level of activity in metaphase II oocytes before fertilization may be because eggs of these two species have larger accumulated maternal stores during these stages of oocyte development.

Our results are comparable with those obtained in *Xenopus* by several research groups. In general, linear DNA is not stable in *Xenopus* oocytes and rapidly degrades although covalently closed circular DNA persists for several days. After fertilization, the amphibian cytoplasm appears to be a more benign environment, since cytoplasmic linear DNA can persist and indeed integrate in about 60% of injected oocytes (Etkin *et al.*, 1984). We have preliminary data indicating that foreign cytoplasmic DNA can integrate in the ovine system (R. M. Moor & D. J. Powell, unpublished observations). Our results may have application for transgenesis in ungulate species and we intend to examine this possibility further by injecting DNA at various stages and at various concentrations to determine the frequency of integration after cytoplasmic injection.

We would like to thank Carol Jennings and Caroline Lee for valuable assistance and Linda Notton for typing the manuscript. The assistance of the Photography Department is also acknowledged.

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Received 1 March 1991