A nuclear DNA Polymerase in bull spermatozoa

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Summary. A DNA polymerase was isolated from bull spermatozoa by differential centrifugation, ultrafiltration and gel filtration. Its apparent molecular weight and synthetic template utilization resemble that of DNA polymerase γ . Chemical and enzymic fractionation of bull spermatozoa indicate that the enzyme is most probably located in the nucleus.

Introduction

Human sperm nuclei contain particulate complexes of high molecular weight RNA and DNA polymerase (Witkin, Korngold & Bendich, 1975; Witkin & Bendich, 1977). While in association with the complex, the enzyme can synthesize DNA in the absence of an exogenous template (Witkin *et al.*, 1975; Bendich, Borenfreund, Witkin, Beju & Higgins, 1976; Witkin & Bendich, 1977). When the complex is disrupted, DNA polymerase activity can be demonstrated in the presence of synthetic deoxy- or ribo-nucleotide polymers (Witkin *et al.*, 1975; Bendich *et al.*, 1976). In the present study, we have isolated and partly characterized a DNA polymerase from bull sperm heads.

Materials and Methods

Bull semen was obtained from Dr G. W. Salisbury, University of Illinois College of Agriculture, and stored at -20°C in 50% glycerol. The semen contained no visible somatic cells or bacteria. Aliquots (25 μl) of semen were stained with Giemsa and the degree of contamination was <400 organisms/ml; it was even less after the centrifugation steps. The spermatozoa were pelleted by centrifugation at 1000 g and resuspended in buffer (0.05 M-Tris-HCl, pH 7.5; 0.15 M-NaCl; 1 mM-EDTA). This procedure was repeated for three cycles to remove seminal fluid contamination. In Exp. 1, the final sperm pellet was resuspended (10^9 spermatozoa/ml, total vol. = 1 ml) in 0.5% sodium lauroyl sarcosinate (Sarkosyl: Chemical Additives Co., Farmingville, New York), 0.05 M-Tris-HCl, pH 7.5, 10 mM-dithiothreitol (Sigma Chemical Co., St Louis, Missouri), and 75 μg trypsin/ml (Worthington Biochemical Co., Freehold, New Jersey). Sarkosyl removes human plasma, acrosomal and nuclear membranes (Witkin, Evenson & Bendich, 1977) and decaudates other spermatozoa (Millette, Gall & Edelman, 1974). The addition of dithiothreitol causes the heads to swell because of reduction of the intranuclear disulphide bridges (Bedford & Calvin, 1974), and then trypsin is able to degrade the chromosomal protein. After incubation at 37°C for 15 min, Trasylol (pancreatic trypsin inhibitor: Mobay Chemical Co., New York) was added (300 kallekrein inhibitory units/ml) to inhibit further trypsin action. The resulting viscous solution was treated with 20 μg deoxyribonuclease/ml (Worthington) and 5 mM-MgCl₂ (final concentration) at 37°C for 15 min to degrade released DNA. Particulate matter, including tail fragments, was then removed by centrifugation for 20 min at 17 000 g . The supernatant was pressure-dialysed in an ultrafiltration cell against a buffer containing 0.05 M-Tris-HCl, pH 7.5, 0.02% Triton X-100, 1 mM-EDTA, 10% glycerol and 0.5 M-KCl (TTK buffer). One-half of the concentrated solution (300 μl) was loaded onto a gel-filtration column (Ultrogel AcA-44; 12 000–130 000 molecular weight range: LKB Instruments Inc., Rockville, Maryland), eluted with TTK buffer, and the fractions were assayed (Weissbach, 1975) for dA_n·dT_{12–18}-templated and rA_n·dT_{12–18}-templated DNA polymerase activities (see Text-fig. 1 for reaction conditions). Subsequently, a 100 μl aliquot of the gel filtration fraction containing

maximal DNA polymerizing activity was layered onto a 10–30% (v/v) glycerol gradient in TTK buffer, centrifuged for 19 h at 165 000 *g* and fractionated. The aliquots were assayed for $rA_n \cdot dT_{12-18}$ -templated DNA polymerase activity.

The procedures described for the washing of the spermatozoa reduced seminal fluid contamination of the cells by a factor of approximately 10^{-10} , but it was possible that any DNA polymerase found was adhering to the sperm membranes or was located in the tail, which contains mitochondria with their associated DNA polymerase (Hecht, 1974). In Exp. 2, therefore, the washed sperm pellet was treated with 1 volume of 0.5% Sarkosyl, 0.05 M-Tris-HCl, pH 7.5, and 10 mM-dithiothreitol and incubated at 37°C for 15 min to solubilize membranes, and remove tails. The swollen sperm heads were then separated from tails and solubilized membranes by centrifugation at 650 *g* for 10 min and resuspended in 10 volumes of Sarkosyl buffer. Differential centrifugation was repeated at 650 *g* for 10 min and the sperm head pellet was resuspended in 1 volume of Sarkosyl buffer. An aliquot removed for examination by phase-contrast microscopy showed that a sperm head population without any tails had been obtained. Trypsin was then added (75 µg/ml) to lyse the sperm heads and the resultant 17 000 *g* supernatant was pressure-dialysed and subjected to gel filtration and velocity centrifugation as described for Exp. 1.

Results

Experiment 1

A large peak of DNA polymerizing activity (Text-fig. 1), able to utilize both synthetic templates but preferring the polyribonucleotide and eluting with a penetration coefficient of approximately zero, was obtained. This elution profile was indicative of a molecular weight of approximately 130 000 or greater. Velocity ultracentrifugation of the active fraction from gel filtration revealed a single peak of activity (Text-fig. 2) which possessed a sedimentation rate in the detergent and high salt-containing TTK buffer of approximately 8S (calculated by using BSA, *S* = 5, as a marker: Martin & Ames, 1961).

Template and divalent cation preferences of this 8S DNA polymerase were determined (Table 1). The enzyme preferred $rA_n \cdot dT_{12-18}$ as the template-primer, and Mn^{2+} was the preferred divalent cation, yielding optimal activity at 1 mM. Mg^{2+} was partly able to replace Mn^{2+} with $rA_n \cdot dT_{12-18}$ as the template-primer, but the polymerizing activity was reduced by 50%. Neither $rC_n \cdot dG_{12-18}$ nor $rC_n^m \cdot dG_{12-18}$ (ribopolymer containing 2'-O-methylcytosine) both of which are reportedly used by viral reverse transcriptase (Gerard, 1975), were utilized by the enzyme.

Table 1. Relative template-primer and divalent cation-dependent activities of the DNA polymerase of bull spermatozoa

Template-primer	Divalent cation	Relative activity*
$rA_n \cdot dT_{12-18}$	Mn^{2+}	1.0
$rA_n \cdot dT_{12-18}$	Mg^{2+}	0.4
$dA_n \cdot dT_{12-18}$	Mn^{2+}	0.65
$dA_n \cdot dT_{12-18}$	Mg^{2+}	0.1
$rC_n \cdot dG_{12-18}$	Mn^{2+}	0
$rC_n^m \cdot dG_{12-18}^\dagger$	Mn^{2+}	0

* Relative amount of DNA polymerase activity, assayed as described in Text-fig. 1. The reaction rate was linear for 45 min; 1.0 corresponds to 120 fmol acid-insoluble product formed per 50 µl reaction mixture.

† This template contains 2'-O-methylcytosine in the ribopolymer.

Experiment 2

The results of this experiment are not given but patterns similar to those shown in Text-figs 1 and 2 were obtained, indicating that the enzyme was most probably located in the sperm nucleus.

Text-fig. 1. Gel filtration of an extract of bull spermatozoa. A 300 μ l sample of the detergent-solubilized and pressure dialysed bull sperm extract was loaded onto a 60 $\times$ 0.9 cm gel-filtration column containing Ultrogel AcA-44 in TTK buffer. The sample was eluted with TTK buffer at atmospheric pressure and a flow rate of 4.8 ml/h. $V_T = 48.6$ ml; $V_0 = 7.2$ ml; effective mol. wt range = 12 000–130 000. Fractions of 300 μ l each were collected and 10 μ l aliquots assayed for (○) $rA_n \cdot dT_{12-18}$ - or (●) $dA_n \cdot dT_{12-18}$ -templated DNA polymerizing activities after combination with 40 μ l of an assay cocktail. The reaction mixture, total volume 50 μ l, contained: 0.32 mM-dATP, 10 mM-dithiothreitol, 1 mM-MnCl₂, 0.02 M-Tris-HCl, pH 7.5, 100 μ g BSA/ml, 9.6 μ g template-primer/ml, and 2.17 μ M-[³H]dTTP (46 Ci/mmol); 2.17 μ M-[³H]dGTP (6 Ci/mmol) were substituted for TTP when $rC_n \cdot dG_{12-18}$ or $rC_n^m \cdot dG_{12-18}$ were used as templates. Incubation was for 15 min at 37°C. The reaction was terminated by addition of 3 ml ice-cold 5% trichloroacetic acid–1% sodium pyrophosphate, and the acid-insoluble product was collected on 25 mm Gelman type A-E filters, dried and counted.

Discussion

The sedimentation rate of the bull sperm head enzyme corresponds to the sedimentation value for DNA polymerase γ from HeLa cells (Spadari & Weissbach, 1974). The template and divalent cation preferences of the sperm enzyme are also consistent with those described for DNA polymerase γ from HeLa cells (Spadari & Weissbach, 1974) but not for RNA-dependent DNA polymerases (reverse transcriptase) (Wu & Gallo, 1975) or DNA polymerases α and β (Spadari & Weissbach, 1974).

The function of DNA polymerase γ remains uncertain (Weissbach, 1975). Nevertheless, the enzyme is unique in that it is able to copy synthetic ribohomopolymers at a higher rate than deoxy-ribomopolymers (Fridlender, Fry, Bolden & Weissbach, 1972). Its purported inability to transcribe natural RNA *in vitro* (Bolden, Fry, Muller, Citarella & Weissbach, 1972) may be an artefact, as suggested by discoveries of ribonuclease-sensitive, endogenous DNA-synthesizing complexes containing DNA polymerase γ isolated from the microsomal fraction of rat brain (Witkin & Schumaker, 1977) and in a nuclear membrane fraction of human lymphoid cells (Yoshida & Cavalieri,

Text-fig. 2. Sedimentation velocity centrifugation of bull sperm DNA polymerase. A 100 μ l aliquot of the peak fraction from the gel filtration column (Text-fig. 1) was layered onto a 5 ml 10–30% (v/v) glycerol gradient in TTK buffer. After centrifugation for 18 h at 165 000 g, 6-drop fractions were collected from the bottom and 10 μ l aliquots were assayed for α_N -dT_{12–18}-dependent DNA polymerase activity as described in Text-fig. 1.

1977). The enzyme may thus be capable of DNA synthetic activities not readily expressed in conventional assay systems. In this regard, measurable DNA polymerizing activity was observed only in sperm preparations after they had been subjected to the gel filtration step described here. It appears that the enzyme may be maintained in the sperm nuclei in an inactive form and that gel filtration helps to remove an inhibitor. Another possibility is that inhibitors of the polymerization reaction are also present in the nucleus, even after removal of the acrosome. The latter possibility does not include DNA degrading enzymes because pressure-dialysed preparations did not exhibit any detectable deoxyribonuclease activity (data not shown).

The association of this inactive DNA polymerase with sperm DNA and its conversion to an active form could facilitate the initiation of repair synthesis at appropriate times before DNA condensation or syngamy. In addition, enzyme activation at some specific stage of spermatogenesis or embryogenesis could allow for selective RNA to DNA transcription of specific gene sequences, thus giving rise to selective gene amplification (Witkin *et al.*, 1975). Experiments are now in progress to test these possibilities.

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