Incorporation of [^3H]thymidine into mtDNA of ram spermatozoa

B. Bartoov, I. Reis and J. Fisher

Department of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel

Summary. Freshly ejaculated spermatozoa from 6 rams incorporated [^3H]-thymidine only after a lag period of 4 h. The thymidine incorporation was dependent on the time after ejaculation regardless of the rate of fructolysis, which was controlled by cold shock. The DNA synthesis period lasted for 3 h followed by a degradation period of 2 h. This synchronized level of labelling of DNA with tritiated thymidine was not due to bacterial contamination, sperm cell mitosis, mitochondrial division, or DNA repair. The fact that the maximal level of the DNA synthesis was dependent upon the external pH of the seminal plasma, in a parabolic fashion with a minimum around pH 6.3, indicated that this activity was of a 'metabolic' type. The sensitivity of the sperm DNA synthesis to 10 μg ethidium bromide/ml indicated that it was of a mitochondrial origin.

Introduction

Ram spermatozoa contain mitochondrial DNA (mtDNA), as well as nuclear DNA (Fisher, Langsam & Bartoov, 1977), which is inactive (Premkumar & Bhargava, 1972). Only the mtDNA, whose properties are similar to its counterparts in somatic cells (Fisher *et al.*, 1977), is active in transcription and translation (Premkumar & Bhargava, 1972; Mujica, 1976; Bragg & Handel, 1979). However, no evidence regarding such mtDNA replication has been reported. The existence of a mitochondrial DNA polymerase of bovine spermatozoa (Hecht, 1974) and of DNase II in the mitochondrial fraction of ram spermatozoa (Fisher & Bartoov, 1980b), both suggest that sperm mtDNA might be engaged in replication.

Since there is a connection between mtDNA replication and cell mitosis, as demonstrated by Pica-Mattoccia & Attardi (1972), Ley & Murphy (1973) and Volpe & Eremenko (1973), the question arises whether mature spermatozoa, which do not undergo mitosis and in which mitochondria do not divide, in fact replicate their mitochondrial DNA, and this was investigated in the present study.

Materials and Methods

Chemicals

Thymidine and RNA from *Torula* yeast Type VI were purchased from Sigma Chemical Company. [Methyl- ^3H]Thymidine (sp. act. 35.2 Ci/mmol) was obtained from Nuclear Research Centre, Negev, Israel. Cefalotin sodium (Keflin) was purchased from E. Lilly Co., and carbenicillin (Pyopen) from Beecham Research Laboratories. Soluene-350 was obtained from Packard Instrument Company, Inc. Ethidium bromide was kindly supplied by Dr S. Lavy, Weizmann Institute, Rehovoth, Israel.

Collection of spermatozoa

Semen was collected from 6 rams by electroejaculation (Bartoov & Messer, 1976), pooled in a sterile Erlenmeyer flask and brought to a final concentration of 0.5×10^9 cells/ml with Ringer–fructose–phosphate buffer, pH 7.4 (p. 347; Mann, 1964). Cefalotin sodium and carbenicillin were added to the cell suspension, each at a concentration of 2 mg/ml, which was preincubated for 30 min at 37°C before the labelling of DNA to kill possible bacteria which might be present in the semen.

Labelling of DNA

All experiments were performed under sterile conditions. All equipment for incubation and sampling was previously sterilized by overnight heating at 180°C. The pH meter electrode was rinsed with 95% ethanol. Between 45 and 75 min after ejaculation, 30 ml of the cell suspension were labelled with 0.4 mCi [methyl- ^3H]thymidine. The mixture was incubated with constant mild shaking at 37°C in a water bath and 0.3 ml aliquots were removed in triplicates, every 30 min after labelling, and placed in 3 ml ice-cold Ringer–fructose–phosphate buffer containing 1 mg unlabelled thymidine/ml. Samples were centrifuged for 10 min at 300 g_{max} and 4°C. Pellets were frozen immediately in liquid N_2 and stored overnight at -70°C . The pH of the incubation mixture was checked at each sampling time, and bacterial contamination was monitored by plating 50 μl samples on brain–heart agar.

Extraction of DNA

The procedure described here is a modification of the hot phenol–sodium dodecyl sulphate (SDS) method described by Penman (1966). Frozen sperm cell pellets were thawed in 2 ml of a solution of 0.025 M-sodium acetate–0.1 M-NaCl, pH 5.0, 1 ml of a yeast RNA solution (10 mg/ml) and 1 ml 20% (w/v) SDS, then shaken on a Vortex mixer. Water-saturated phenol (2 ml) was added and the mixture shaken again. The mixture was heated to 60°C for 2 min and shaken again before addition of 2 ml chloroform containing 1% isoamyl alcohol, reheating to 60°C for 2 min and further shaking. After centrifugation at room temperature and 1650 g_{max} for 10 min, the lower phenol–chloroform phase was removed with a Pasteur pipette. The procedure was then repeated using the chloroform–isoamyl alcohol mixture alone. The aqueous phase from the last extraction was placed in 1 ml ice-cold yeast RNA solution (10 mg/ml) and 5 ml ice-cold 10% trichloroacetic acid were added. The suspension was mixed and centrifuged at 5000 g_{max} for 10 min at 4°C. The precipitate was washed with 5 ml ice-cold 5% trichloroacetic acid, solubilized overnight with 0.5 ml solouene-350, and added to dioxan–naphthalene liquid scintillant (Bartoov, Mitra & Freeman, 1970) for counting of the radioactivity in a liquid scintillation counter.

Treatment of cells

Mild cold shock. Fructolysis is irreversibly inhibited by a sudden cooling of ejaculated semen (p. 357, Mann, 1964) and the samples were therefore mildly cold shocked to reduce the amount of fructolysis. The cell suspension (75 ml) was transferred with the flask from a 37°C to an ice-bath for 30 sec (reached 15°C), then replaced in the 37°C water bath for 2 min (reached 34°C) and again into the ice bath for another 30 sec (reached 15°C) and returned to the 37°C bath. This mild cold shock did not cause any motility inhibition and occasionally motility was stimulated.

Ethidium bromide treatment. The sensitivity of [^3H]thymidine incorporation into DNA was examined by mixing the cell suspension with a freshly prepared ethidium bromide stock solution (10 mg/ml) to give a final concentration of 10 μg ethidium bromide/ml.

Results

Level of labelling of DNA with tritiated thymidine

The results of 9 [^3H]thymidine incorporation experiments (from 5 different ejaculations) showed that DNA was synthesized by freshly ejaculated ram spermatozoa (Text-fig. 1). There was a relatively long period of delay, usually about 4 h from ejaculation, a sharp increase for about 3 h, a rapid degradation over about 2 h, and a variable maximum level of thymidine incorporation (Table 1).

Text-fig. 1. Time course of [^3H]thymidine incorporation into mtDNA by freshly ejaculated ram spermatozoa in 4 experiments. Each sample of 15×10^9 cells was suspended in 30 ml Ringer–fructose–phosphate buffer, labelled with 0.4 mCi [^3H]thymidine and incubated at 37°C. Aliquots were taken every 30 min and DNA isolated, as described in ‘Materials and Methods’.

■, ●, Untreated cells; □, ○, mild cold shock-treated cells.

Effect of pH

Freshly ejaculated spermatozoa continuously produce lactic acid, due to fructolysis, causing a pH drop of the seminal plasma (p. 88; Mann, 1964). The rate of the pH drop depends upon the rate of fructolysis, which varies from one experiment to another and is reduced by a mild cold shock. This mild cold shock treatment, although reducing fructolysis, did not affect the level of labelling of DNA (Text-fig. 2). No correlation was found between the pH of the plasma and the start of the synthesis ($r = -0.594$) or degradation ($r = -0.271$) (Table 1). However, the maximum level of [^3H]thymidine incorporation into DNA was dependent on the pH of the plasma in a parabolic fashion (Text-fig. 3).

Inhibition by ethidium bromide

Incorporation of [^3H]thymidine into DNA was inhibited by 10 μg ethidium bromide/ml (Text-fig. 4).

Table 1. Labelling of DNA in freshly ejaculated ram spermatozoa with [³H]thymidine

		Synthesis phase				Degradation phase	
Ram group	Sample	Time from ejaculation (min)		Duration (min)	Maximum [³ H]thymidine incorporation (c.p.m./10 ⁹ cells)	Time from ejaculation to end (pH)	Duration (min)
		Start (pH)	End (pH)				
I	1	255 (5.7)	465 (5.7)	210	4590	570 (5.7)	105
	2*	255 (6.7)	495 (6.7)	240	9564	570 (6.7)	75
II	3	280 (6.3)	370 (6.3)	90	3253	475 (6.0)	105
	4*	280 (6.5)	425 (6.5)	145	3373	520 (6.2)	95
III	5	340 (5.7)	490 (5.4)	150	12 550	480 (5.4)	90
	6*	340 (6.6)	490 (6.6)	150	9375	580 (6.4)	90
IV	7	150 (7.0)	325 (6.6)	175	7046	535 (5.4)	210
	8*	150 (7.2)	335 (7.2)	185	14 600	455 (6.8)	120
V	9	65 (6.7)	290 (6.0)	225	3410	515 (5.2)	225
Mean ± s.e.		235 ± 31	409 ± 27	174 ± 15		533 ± 15	123 ± 18

* These samples were subjected to mild cold shock (see text).

Text-fig. 2. Time course of [³H]thymidine labelling in DNA and the pH drop (-----), due to fructolysis, of untreated (●) and mild cold shock-treated (○) cells from the same ejaculate. Experimental conditions were as for Text-fig. 1.

Discussion

The results presented in this paper indicate for the first time that ram spermatozoa can incorporate tritiated thymidine into their DNA. The signal for DNA synthesis takes place after a lag period of about 4 h from semen ejaculation. The DNA synthesis phase is limited to about 3 h and then there is a rapid degradation phase of 2 h resulting in the complete breakdown of the previously synthesized DNA.

The sensitivity of the DNA synthesis to ethidium bromide indicates that the DNA synthesis is probably of mitochondrial rather than nuclear origin (Nass, 1970, 1972), as would be

Text-fig. 3. The relationship between the pH of the seminal plasma and the maximum level of [^3H]thymidine incorporation at the end of the DNA synthesis phase. Untreated sperm cells (●); mild cold shock-treated sperm cells (○).

Text-fig. 4. The effect of ethidium bromide (10 $\mu\text{g}/\text{ml}$) on the [^3H]thymidine incorporation into DNA of ram spermatozoa (○, untreated; ■, treated).

expected from the reported inactivity of nuclear DNA of mammalian spermatozoa (Premkumar & Bhargava, 1972). Further indirect evidence for the possible replication of mtDNA in ejaculated ram spermatozoa is provided by the observation of replicative intermediates as double-forked circular DNA molecules from an isolated ram sperm mitochondrial fraction (Fisher & Bartoov, 1980a).

The labelled DNA was extracted from frozen cell pellets rather than from isolated mitochondrial fractions for the following reasons. (1) To obtain a reasonable amount of final

mitochondrial fraction (about 2.5 mg protein) 10^{10} spermatozoa are required, and to follow the kinetics of DNA synthesis and degradation during a 10-h experiment, from the time of ejaculation, it would be necessary to collect semen from about 50 rams. (2) Each isolation of sperm mitochondria should be from fresh spermatozoa, a procedure which requires about 5 h, thus making a long kinetic study of [^3H]thymidine incorporation technically impossible. (3) The mtDNA isolated from mitochondrial fraction is accessible to intensive DNase activity, as reported by Fisher *et al.* (1977) and Fisher & Bartoov (1980b). Thus, some of the [^3H]thymidine DNA can be lost by enzymic degradation. A more direct proof for the mitochondrial origin and characterization of the synthesized DNA could be obtained by electron microscope autoradiography on replicative intermediates of circular mtDNA molecules and this study is planned.

Several lines of evidence argue against the synthesis being due to bacterial contamination, since the [^3H]thymidine incorporation experiments were performed under strict sterile conditions, incubated in the presence of antibiotics, and checked continuously for bacterial growth. The fact that the mtDNA synthesis occurs a considerable time after ejaculation and the rapid degradation at a particular time after [^3H]thymidine incorporation also argue against bacterial contamination.

The marked synthesis and degradation of sperm DNA in relation to the time of ejaculation indicate a synchronization which may be controlled by internal or external factors. The absence of correlation between the signals for DNA synthesis or degradation and the pH of the plasma excludes pH, an indicator of the intensity of fructolysis, as being a synchronizing factor. Nevertheless, the maximum level of [^3H]thymidine incorporation was influenced by the pH of the seminal plasma in a parabolic fashion with a minimum around pH 6.3. The newly synthesized mtDNA in ram spermatozoa therefore seems to be of the "metabolic-type" (Pelc, 1968).

The mtDNA can be active in three ways, similar to those described for DNA in general by Davidson (1972): (a) premitotic synthesis, as reported in many mammalian cells, such as cultured human Chang liver cells (Koch & Stockstad, 1967), mouse L cells (Nass, 1967), mouse lymphoma cells (Bosmann, 1971) and human HeLa cells (Pica-Mattoccia & Attardi, 1972; Volpe & Eremenko, 1973), with the maximum rate of mtDNA synthesis in the G_2 phase, with some synthesis in the S phase; (b) repair, as demonstrated by Keiding & Westergaard (1971) in *Tetrahymena* after u.v. irradiation; and (c) formation of metabolic DNA, as indicated by Georgatsos, Taylor-Papadimitriou & Karemflyllis (1972) and Dallman (1971). In sperm cells, however, mtDNA synthesis cannot be related to the first two activities because sperm cells do not undergo mitosis and their mitochondria do not divide, and the sperm level of labelling of mtDNA with [^3H]thymidine is characterized by a synchronized synthesis followed by a sharp decline, a phenomenon which cannot be explained in terms of repair activity.

It is possible that the synchronized synthesis and degradation of mtDNA of ram spermatozoa is connected with a regulating mechanism governing energy metabolism. Generally, mature ejaculated ram spermatozoa utilize the fructolytic pathway and mitochondrial oxidative phosphorylation simultaneously as energy sources for their motility (Rikmenspoel, Sinton & Janick, 1969). However, no Pasteur effect was observed in ejaculated mammalian spermatozoa when examined under aerobic conditions (p. 299; Mann, 1964) or in the presence of 2,4-dinitrophenol (Peterson & Freund, 1969). It is therefore possible that the relation between these two sources of energy production is regulated by the mtDNA gene product. Another possible regulatory effect of the mtDNA could be on the ratio between the amount of fructose or glucose utilized to produce lactic acid and that converted to carbon dioxide within the mitochondria. Voglmayr, Scott, Setchell & Waites (1967) have shown that ram ejaculated spermatozoa utilized about 90% of the glucose uptake into lactic acid, but this percentage changed with time and was dependent upon the medium. The minimal intensity of mtDNA synthesis at pH 6.3 reported herein, and the maximum respiration of washed bull

spermatozoa at the same pH (Cragle & Salisbury, 1959) seem to point to a possible regulatory relation between the mtDNA and mitochondrial respiration.

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