Study of dogfish (*Scyliorhinus caniculus*) deoxyribonucleic acid polymerases α and β

Extraction, separation, characterization and changes during spermatogenesis

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DNA polymerase activity was extracted from testis cells of the dogfish *Scyliorhinus caniculus*. On a sucrose gradient, two main peaks could be separated, corresponding to DNA polymerases β (3.8 S) and α (7.5 S). DNA polymerase γ could also be detected when poly(A)·(dT)₁₂ was used as template. The properties of α and β polymerases of this primitive vertebrate were similar to those generally described, especially in mammals. The β enzyme was highly sensitive to *N*-ethylmaleimide, however, and could use poly(dT)·poly(A) as template. Polymerase α was present in spermatogonia, spermatocytes and spermatids. Activity was maximal in spermatocytes. DNA polymerase β was present in all testis cells with similar activities in spermatogonia and spermatocytes. Decreased activities were observed during spermiogenesis. Some activity remained associated with the chromatin fraction of mature sperm cells.

Three species of DNA polymerase, α , β and γ , have been isolated from mammalian cells and their biochemical characteristics are described in several reviews (Craig & Keir, 1974; Fansler, 1974; Bollum, 1975; Weissbach, 1977; Sarngadharan *et al.*, 1978).

In an attempt to attribute a definite role to each polymerase species *in vivo*, work has been carried out on differentiating cell systems: muscle cells (Grippe *et al.*, 1977), brain neurons, cardiac muscle and spleen differentiation during perinatal development (Hübscher *et al.*, 1977), concanavalin A-stimulated spleen cells (Spadari *et al.*, 1978) and developing testis cells (Hecht *et al.*, 1976).

Spermatogenesis appears to be a good experimental system for this type of study, since DNA replication occurs principally in spermatogonia and DNA repair is undoubtedly required after recombination in spermatocytes. We previously used cytoenzymological techniques to study variations of nuclear DNA polymerase activity during spermatogenesis in mouse testes (Chevaillier & Philippe, 1976). Increased activity was found in meiotic prophase, followed by a decrease in spermatids and spermatozoa, and these results were confirmed with spermatogenic cells separated by velocity sedimentation (Daentl *et al.*, 1977). By using the same cell system and the same techniques, it was demonstrated that DNA polymerase α could be detected only in the fraction enriched in

spermatogonia and in preleptotene spermatocytes; β -enzyme activity, on the other hand, was present in all cells; its activity reached a peak at middle-late pachytene and activity subsequently decreased during spermiogenesis (Grippe *et al.*, 1978).

We adopted dogfish (*Scyliorhinus caniculus*) testes as our experimental system, since germ cells are contained within follicles and all the cells in a given follicle are in the same developmental stage. In addition, follicular genesis is continuous, i.e. newly formed follicles migrate away from the germinal sites as they mature along the testis and are replaced by successively more juvenile elements (Stanley, 1966). It thus becomes possible to select follicles at the same developmental stage. Even though this system is well adapted to our studies because of the natural synchrony of the cells, very little is known about the various DNA polymerase activities in this vertebrate group. We report here the partial characterization of two DNA polymerase species after their separation on sucrose gradients. Variations of activity as a function of the stage of spermatogenesis were also determined.

Experimental

Dogfish were provided by the 'Station Biologique', Roscoff, France.

For preparation of cell fractions, follicle zones

corresponding to synchronous cells were selected from testicular sections and were verified by microscopic examination. For light-microscopy, samples of all cell fractions were squashed on the slide and DNA was stained as described by Feulgen & Rossenbeck (1924). Electron microscopy was done as described by Philippe & Chevaillier (1978a).

For preparation of extracts, follicular fractions were homogenized in 10mM-Tris/HCl (pH 7.5)/10mM-KCl/1.6mM-MgCl₂/0.5mM-dithiothreitol (Hübscher *et al.*, 1977), or in 0.2M-potassium phosphate buffer (pH 7.5)/2mM-EDTA/2mM-2-mercaptoethanol (Mechali *et al.*, 1977). Both media contained 2M-KCl, 0.5% Triton X-100 and 100 µg of soya-bean trypsin inhibitor/ml. Cells were broken by ultrasonication with the micro-probe of a MSE Ultrasonicator at maximum power for 2 × 10 s. Extraction was for 2 h at 4°C with occasional agitation. The extracts were clarified by centrifuging with a SW 50.1 rotor for 1 h at 40000 rev./min. Some extracts were then concentrated with an Amicon B15 concentrator.

Isokinetic sucrose gradients were used to separate the different species of DNA polymerase and to determine their sedimentation coefficients (Martin & Ames, 1961). The technical procedure is extensively described by Philippe & Chevaillier (1978b). The following proteins were used as sedimentation markers: ovalbumin (3.6S), alcohol dehydrogenase (7.4S) and ox liver catalase (11.3S) (Boehringer, Mannheim, West Germany).

DNA polymerase activity was measured with activated DNA and with different synthetic templates in order to distinguish polymerase- α , - β and - γ activities. Material and experimental conditions are described in detail by Philippe & Chevaillier (1978b).

DNA was detected colorimetrically by the diphenylamine method (Burton, 1956).

Results and discussion

The initial parameter investigated was the total extraction of DNA polymerase activity from testis cells. Similar results were obtained when two different extraction media were used (Hübscher *et al.*, 1977; Mechali *et al.*, 1977), but sonication was shown to be required. An endogenous activity was found in extracts, which was greater after sonications. This phenomenon probably corresponded to the shearing of chromatin into small soluble fragments.

The two major peaks at 7.5S and 3.8S on sucrose gradients correspond to findings of Chang (1976), who termed these species, extracted from various animal organisms, α and β . In some cases the lighter peak appeared to be heterogeneous, but could not be dissociated at higher speeds and longer centri-

fugation times, e.g. 20 h at 50000 rev./min in the SW 50.1 rotor.

Both activities required Mg²⁺, with optimal concentrations between 5 and 10mM. The replacement of Mg²⁺ by Mn²⁺ led to dramatic losses of activity, similar to observations with mammalian DNA polymerases α and β (Baril *et al.*, 1973; Craig & Keir, 1974; Fansler, 1974; Bollum, 1975; Weissbach, 1977; Sarngadharan *et al.*, 1978). Similarly, the sensitivity of the α enzyme to univalent cations, with activity increasing up to 40mM-K⁺ and up to 20mM-Na⁺, followed by a dramatic decrease at higher concentrations, and the resistance of the β enzyme up to concentrations of 500mM-Na⁺ or -K⁺, are consistent with the properties of other α and β polymerases (Craig & Keir, 1974; Fansler, 1974; Bollum, 1975; Weissbach, 1977; Sarngadharan *et al.*, 1978). Enzyme activities were found to depend on both pH and buffer composition. Thus, the α enzyme was stimulated by potassium phosphate buffer (20mM, pH 7.2), whereas the β enzyme was inhibited in this buffer; its optimal activity was found at higher pH (50mM-borate/NaOH, pH 9.15, or 50mM-Tris/HCl, pH 8.8). This is also in agreement with published results on α and β polymerases (Craig & Keir, 1974; Fansler, 1974; Bollum, 1975; Weissbach, 1977; Sarngadharan *et al.*, 1978).

As shown in Table 1, dithiothreitol, which has no effect on polymerase β , was found to be a powerful activator of the α enzyme. *N*-Ethylmaleimide inhibited both α and β enzymes from dogfish; such a result was also found for catfish (*Silurus* sp.) cells in culture (A.-M. de Recondo, personal communication). A high sensitivity to *N*-ethylmaleimide has been reported also for β -polymerase, not from fish but from insects (Chang, 1976), and work in our laboratory showed that the β enzyme extracted from bull spermatozoa was partially sensitive to this inhibitor, depending on the concentration (Philippe & Chevaillier, 1978a). Other cases of β -polymerase sensitivity to *N*-ethylmaleimide are cited in that paper. The activities of α and β polymerases were relatively more resistant to ethidium bromide than was mitochondrial DNA polymerase, where more than 80% of mitochondrial activity was inhibited by 16 µM-ethidium bromide (Hecht, 1974). At higher concentrations α and β polymerases were sensitive, but the α enzyme was more sensitive; this is in agreement with other results (Kornberg, 1974). Both enzymes were inhibited by phosphonoacetic acid; the greater sensitivity of the α enzyme is consistent with data obtained with other mammalian systems (Bolden *et al.*, 1975; Allaudeen & Berino, 1978).

Both polymerases used activated DNA and poly(dT)·poly(dA) as templates. Poly(dT)·poly(A) is considered to be a specific template for the α

EXPLANATION OF PLATE 1

Microscopy of testis fractions I-III

Fraction I (spermatogonia): (a), light-microscopy (magnification $\times 720$); (b) electron microscopy (magnification $\times 12000$). Fraction II (spermatocytes): (c), light-microscopy (magnification $\times 720$); (d) electron microscopy (magnification $\times 9600$). Fraction III (round spermatids): (e), light-microscopy (magnification $\times 720$); (f), electron microscopy (magnification $\times 12000$).

EXPLANATION OF PLATE 2

Microscopy of testis fractions IV and V

Fraction IV (elongated spermatids): (a), light-microscopy (magnification $\times 720$); (b) and (c), electron microscopy (magnification $\times 12000$). Fraction V (spermatozoa): (d), light-microscopy (magnification $\times 720$); (e), electron microscopy (magnification $\times 9600$).

Table 1. *Influence of various activators or inhibitors on DNA polymerases α and β*
For details see the Experimental section.

		Activity (% of control)	
		Polymerase α	Polymerase β
Control		100	100
+ Dithiothreitol (mM)	0.5	226	98.5
	1	259	102
	5	297	95.5
	10	283	94.5
+ <i>N</i> -Ethylmaleimide (mM)	0.1	26.3	38
	0.5	13.7	22
	1	7.5	15
+ Ethidium bromide (μ M)	10	62	75
	25	45	52
	50	14	23
+ Phosphonoacetic acid (μ g/ml)	1	101.5	94.7
	5	67.1	74.5
	10	51	68
	50	23.4	47

enzyme (Craig & Keir, 1974; Fansler, 1974; Bollum, 1975; Weissbach, 1977; Sarngadharan *et al.*, 1978). In our case, both enzymes were active with this substrate. The only other β enzyme capable of using this polynucleotide as template is that from *Xenopus laevis* ovaries (Joenje & Benbow, 1978). Like the *Xenopus laevis* β polymerase, our β enzyme was more active with poly(A)·(dT)₁₂ than with activated DNA. In the presence of phosphate buffer and poly(A)·(dT)₁₂ only the γ enzyme retained activity. This agrees with published properties of mammalian β and γ enzymes (Change, 1974; Knopf *et al.*, 1976).

Five maturational stages, selected from testis cross-sections, were identified by microscopy: I, spermatogonia (Plates 1a and 1b); II, spermatocytes (Plates 1c and 1d); III, round spermatids (Plates 1e and 1f); IV, elongated spermatids (Plates 2a, 2b and 2c); V, old spermatids and spermatozoa (Plates 2d and 2e). Extracts of each fraction were prepared and centrifuged on isokinetic sucrose gradients. By using the appropriate templates and incubation conditions, DNA polymerase activities were assayed in the crude extracts (Table 2) and in the fractions recovered from the gradients (Fig. 1).

With activated DNA as template, maximum activity was found in spermatocytes (Table 2), dramatically decreasing as spermiogenesis progressed. This is in agreement with results obtained in our laboratory by using cytoenzymology on thin sections of mouse testes (Chevaillier & Philippe, 1976) and with other results on mouse spermatogenic cells separated by velocity sedimentation (Daentl *et al.*, 1977; Grippo *et al.*, 1978; Hecht *et al.*, 1979). However, not in good

accordance with prior work (Chevaillier & Philippe, 1976), in the present case we found less activity in spermatids and spermatozoa than in spermatocytes. This may be partially due to the fact that the total extraction of DNA polymerase from spermatids and spermatozoa is difficult as a result of the highly condensed structure of chromatin in these cells (Philippe & Chevaillier, 1978a). In the dogfish, histones are replaced by protamine-like proteins during spermiogenesis, and the latter provoke chromatin condensation (Gusse & Chevaillier, 1978).

In agreement with Hecht *et al.* (1979), we detected (Fig. 1) the α enzyme in spermatogonia, spermatocytes and round spermatids (maximum amount in spermatocytes), whereas Grippo *et al.* (1978) found the α enzyme only in spermatogonia and preleptotene spermatocytes. In addition, we detected (Fig. 1) the γ enzyme in all extracts, whereas Grippo *et al.* (1978) did not report its presence. It has been demonstrated that nuclear and mitochondrial DNA polymerases are present in ejaculated mammalian spermatozoa (Hecht, 1974; Witkin & Bendich, 1977; Witkin *et al.*, 1977; Philippe & Chevaillier, 1978a,b). We also found (Fig. 1) that β -enzyme amounts were similar in spermatogonia and spermatocytes, whereas Grippo *et al.* (1978) reported that β -enzyme amounts were maximum in early-pachytene spermatocytes. Comparisons of our results with those of others (Grippo *et al.*, 1978; Hecht *et al.*, 1979) must nonetheless be undertaken with caution. For one thing, there are differences in the biological material. In addition, contamination of a given cell fraction by other cells is undoubtedly highly dependent on the

Table 2. *Variations of DNA polymerase activity in the different cell extracts*

The DNA content was determined in each extract and the results correspond to the same quantity of DNA in each fraction. Activities are expressed in μmol of labelled precursor incorporated in 10min with each template activity, measured as described in the Experimental section. Values in parentheses are percentages of maximum values.

	Template	...	Activated DNA	poly(A)·(dT) ₁₂		Poly(dT)·poly(A)
				+ 50mM-Tris/HCl, pH 7.5	+ 20 mM-potassium phosphate, pH 7.2	
Fraction						
I			0.765 (43)	2.146 (100)	0.246 (100)	0.205 (46)
II			1.759 (100)	2.034 (95)	0.215 (87)	0.446 (100)
III			0.413 (23)	1.345 (63)	0.198 (81)	0.149 (33)
IV			0.279 (16)	0.227 (11)	0.073 (30)	0.076 (17)
V			0.141 (8)	0.215 (10)	0.072 (29)	0.029 (7)

Fig. 1. *DNA polymerase activity in various cell fractions with different templates*

Extracts were prepared corresponding to each cell fraction: spermatogonia (a,f), spermatocytes (b,g), round spermatids (c,h), elongated spermatids (d,i) and spermatozoa (e,j). They were centrifuged for 15.5 h at 192000 g (SW 50.1 rotor) on a 5–20% (w/w) linear sucrose gradient in 50 mM-Tris/HCl (pH 8.3)/0.5 mM-dithiothreitol/0.5 M-KCl. Ovalbumin (3.6S; ▼) and alcohol dehydrogenase (7.4S; ▽) were used as markers. Fractions were collected and DNA polymerase activity was measured in each fraction with different templates and expressed in μmol of labelled precursor incorporated in 10min. The DNA content was determined in each extract and the results correspond to the same quantity of DNA in each fraction. Templates: (a)–(e), activated DNA (●) and poly(dT)·poly(A) (○); (f)–(j), poly(A)·(dT)₁₂ in the presence of 50 mM-Tris/HCl buffer (■) or 20 mM-potassium phosphate buffer (□).

isolation method used. It is also important to determine the exact stage of the spermatocytes in question, since it has been shown (Hecht, 1972) that DNA polymerase activity is variable during meiosis. Thus, in the present case all the cells in fraction II were spermatocytes, but this fraction is clearly heterogeneous.

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