RELEASE OF CHROMATIN TEMPLATE RESTRICTION IN RABBIT SPERMATOZOA

W.D.W. Heston[1], Barry R. Zirkin[2], and Donald S. Coffey[3]

Department of Pharmacology and Experimental Therapeutics
The Johns Hopkins University School of Medicine[1],
Department of Population Dynamics,
The Johns Hopkins University School of Hygiene[2],
and The Brady Laboratory of Reproductive Biology,
The Johns Hopkins University School of Medicine[3]
Baltimore, Maryland 21205

Received March 12,1975

SUMMARY. The addition of the acidic polymers heparin or polyxanthylic acid to rabbit spermatozoa or sperm heads previously exposed to disulfide reducing agents released sperm DNA template restriction and stimulated high levels of incorporation of DNA precursor into DNA, as assayed with exogenous DNA polymerase. Incorporation did not occur in the presence of DNAase, or in the absence of magnesium ion, any of the four deoxyribonucleotides, or E. coli DNA polymerase. This represents the first report that spermatozoa can synthesize DNA in vitro.

INTRODUCTION. The chromatin of eutherian sperm nuclei is entirely repressed with respect to both DNA and RNA synthesis (1). Several hours after fertilization, however, DNA template restriction in the fertilizing sperm nucleus is released and DNA synthesis ensues (2). The factors in the egg cytoplasm which control the release of DNA template restrictions have not been resolved.

Previous work has shown that the addition of specific acidic polymers to isolated somatic cell nuclei or chromatin results in the increase of DNA template availability for DNA or RNA synthesis (3). Release of DNA template restriction appears to involve the interaction of acidic polymers with basic proteins of chromatin (4). This causes the destabilization of nucleohistone and results in the unmasking of the DNA template (4).

In an effort to determine the characteristics of the factors involved in DNA template release in the fertilizing sperm nucleus, we have initiated studies of the effects of acidic polymers on DNA synthesis in ejaculated rabbit spermatozoa. In this paper, we present evidence that the addition of certain acidic polymers to rabbit sperm nuclei, during or after exposure of the spermatozoa to disulfide reducing agents, results in stri-

king stimulation of DNA synthesis in the presence of exogenous E. coli DNA polymerase.

METHODS. Sperm ejaculates were obtained from New Zealand white rabbits by means of an artificial vagina. Ejaculates containing visible amounts of urine were discarded. Several (5-8) ejaculates were pooled, brought up to a volume of 40 mls. with TKM buffer (50mM Tris; 50mM KCl; 5mM MgCl2; pH 7.4) and centrifuged at 1000xg for 10 minutes. The pellet was washed twice by resuspension in 40 mls. of TKM and centrifugation at 1000xg for 10 minutes. This pellet was resuspended in 2 mls. of 50mM Tris buffer at pH 8.0, and then divided into two 1 ml. aliquots, one of which was treated with thiol reagents and the other of which served as control. To the first 1 ml. aliquot, 1 ml. of 10mM dithiothreitol and 3 mls. of 50mM Tris (pH 8.0 were added. This mixture was kept on ice for 15 minutes, after which an additional 4 mls. of Tris (pH 8.0) and 1 ml. of 10% Triton X-100 were added. This final mixture was kept on ice for an additional 45 minutes. Following this incubation in thiol reagent, the sperm were diluted to 40 mls. with TKM buffer (pH 7.4) and were centrifuged at 1000xg for 10 minutes. The pellet was then washed in 40 mls. of TKM buffer. This final sperm preparation is referred to as "thiol-treated". The second 1 ml. aliquot of spermatozoa served as control and received similar treatment but without the presence of dithiothreitol and Triton.

The final pellets for both thiol-treated and control spermatozoa were resuspended in TKM to obtain a final concentration of DNA of 0.5 mg/ml. DNA concentrations were determined by Burton's modification of the diphenylamine method (5) using calf thymus DNA as a standard. Nucleic acids were extracted from the various preparations by the methods of Hutchison et al (6).

Where indicated, separation of sperm heads from midpieces and tails was achieved on a discontinuous sucrose gradient after sonication. The methods used were modified from Stambaugh and Buckley (7) and Hecht (8). Sonication was accomplished with a Branson Ultrasonics Cell Disruptor (Model W140) operating at 4 amps. Sperm were suspended in 5 mls. of cold TKM and sonicated with four 30 sec. intervals and intermittent 1 min. cooling periods. Phase-contrast microscopy indicated 95% sperm head and midpiece-tail separation. The sonicated sperm suspension was diluted 1:1 with 2M sucrose-TKM and layered over 10 mls. of 2M sucrose-TKM. This preparation was centrifuged for 1.5 hrs. at 10,000xg in a Sorvall HB4 rotor. The tails were collected from the interface and were washed twice with 40 mls. of TKM. The pelleted heads were washed in the same manner. Phase-contrast microscopy showed less than 5% contamination in both fractions.

Liver nuclei were prepared from adult male Sprague-Dawley rats (250-300g) by the heavy sucrose method of Blobel and Potter (9). Cytological examination revealed insignificant cytoplasmic contamination. The final purified nuclei were suspended in TKM buffer at a concentration of 1 mg. DNA/ml.

Highly polymerized calf thymus DNA was dissolved in 0.015M NaCl-0.0015M sodium citrate at a concentration of approximately 1mg DNA/ml. The DNA was denatured by heating to 100°C for 10 min. and then rapidly cooled in an ice bath.

DNA template activity was measured by the DNA-dependent incorporation of radioactivity from [^{3}H-methyl]TTP into acid-insoluble material. The constitutents of the reaction system were the following:

A 0.1ml reaction system contained 10μmoles tris-HCl, pH 7.4 at 37°C; 0.7 μmoles $MgCl_2$; 0.1 μmole 2-mercaptoethanol; 18.7 nmoles each of dCTP, dGTP, dATP;20 nmoles of TTP containing 1μCi of [^{3}H]TTP; 0.6 unit of E. coli DNA polymerase (E.C.2.7.7.7); 10.0 μg of the appropriate DNA template as indicated; and an ATP-generating system containing 0.25 μmole ATP, 0.50 μmole sodium phospho(enol) pyruvate, and 0.4μg pyruvate kinase. The system was incubated at 37°C for 60 minutes and assayed by the filter paper disk procedure (10). After appropriate washing, the radioactivity on the disk was determined by scintillation counting.

Highly polymerized calf thymus DNA, heparin, TTP, dCTP, dATP, dGTP, ATP, PEP, pyruvate kinase, dithiothreitol, and 2-mercaptoethanol were purchased from Sigma. [^{3}H-methyl]TTP (sp. act. 53.7 Ci/mmole) was purchased from New England Nuclear. E. coli DNA polymerase (deoxyribonucleotide triphosphate: DNA deoxynucleotidyltransferase, E.C.2.7.7.7, fraction VII, 5000 units/mg.) was purchased from Biopolymers. The polyribonucleotides were all of molecular weight greater than 100,000 and were purchased from Miles Laboratories. Electrophoretically purified pancreatic DNAase 1 (2,900 units/mg.) was purchased from Worthington.

RESULTS AND DISCUSSION. Intact spermatozoa or sperm heads do not serve as effective DNA template sources in a DNA polymerase assay system. However, DNA template restrictions can be removed if the sperm are treated with disulfide reducing agents and then, or simultaneously, with exogenous acidic polymers such as heparin or polyxanthylic acid (Table 1). Polycytidylic acid, which possesses the same net negative charge as polyxanthylic acid, is ineffective in releasing DNA template. Similarly, heparin and polyxanthylic acid, but not polycytidylic acid, increase DNA template activity when added to isolated rat liver nuclei. Disulfide reduction is not mandatory for the release of nuclear DNA template restrictions with liver nuclei. In comparison, purified, heat-denatured calf thymus DNA is an effective template without added acidic polymers; the presence of acidic polymers does not further increase template activity and, in fact, as seen with heparin, acidic polymers can have an inhibitory effect. The results obtained with rat liver nuclei and calf thymus DNA also have been reported previously (3).

The requirement for disulfide reducing agents with rabbit spermatozoa, but not with rat liver nuclei, results from the high content of disulfide crosslinks in eutherian sperm chromatin (11-13). Almost all of the sulfhydryl groups of the large numbers of cysteine residues in eutherian sperm chromatin form disulfide crosslinks during the maturation of spermatozoa in the epididymis (14-16). Apparently, some of all of these crosslinks must be broken in order to activate the sperm DNA template. The possibility that disulfide crosslinks must be broken during normal fertilization has been suggested previously (17).

TABLE I

Comparison of DNA Template Activity of Rabbit Sperm, Rat Liver Nuclei, and Purified Calf Thymus DNA in the Presence of Various Acidic Polymers

	SOURCE OF DNA TEMPLATE (equivalent to 100µg DNA/ml)			
	Rabbit Spermatozoa			
Additions	Thiol-treated	Control	Rat Liver Nuclei	Calf Thymus heat-denatured DNA
	[^{3}H] dTMP incorporation:cpm/hr.			
none	335	152	210	4550
Heparin	1500	157	2590	1270
Poly X	1440	398	2450	3450
Poly C	332	180	280	4720
2-mercaptoethanol (MSH), 10mM	472	154	230	4140
Heparin + MSH	3900	2150	3940	-
Poly X + MSH	2870	668	-	-
Poly C + MSH	516	218	-	-

A 0.1ml reaction system contained 10µmoles tris-HCl, pH7.4; 0.7µmole $MgCl_2$; 0.1µmole 2-mercaptoethanol; 18.7 mµmoles each of dCTP, dGTP, dATP; 20.0 mµmoles of TTP containing 1µCi of [^{3}H]TTP; 0.6 unit of E. coli DNA polymerase; 10µg of the appropriate DNA template as indicated; 1µmole 2-mercaptoethanol where indicated; 10µg of neutralized acidic polymer where indicated; and an ATP generating system containing 0.25µmole ATP, 0.50µmole sodium phospho (enol) pyruvate and 0.4µg pyruvate kinase. The system was incubated at 37°C for 60 minutes and assayed by the filter paper disk procedure. After appropriate washing, the radioactivity on the disk was determined by scintillation counting. The synthetic polyribonucleotides exhibited a molecular weight by gel filtration>100,000.

Thiol-and heparin-treated spermatozoa were tested under various assay conditions to determine the characteristics of the reaction end product (Table 11). Incorporation of [^{3}H]dTMP requires all four deoxyribonucleotides, magnesium ion, and the DNA polymerase enzyme. In addition, incorporation is abolished by the presence of DNAase 1. These observations indicate that incorporation of [^{3}H]dTMP is into DNA and does not represent

TABLE II

Characteristics of DNA Polymerase Activity with Rabbit Sperm DNA as a Template Source

System	Template source equivalent to 100μg DNA/ml; treated sperm + MSH + Heparin 100μg/ml. [^{3}H]dTMP incorporation:cpm/hr.
Complete	3900
omit Sperm DNA	0
omit $Mg^{(++)}$	0
omit dATP	241
omit dCTP dGTP	520
omit DNA Polymerase	0
Deoxyribonuclease[1]	0

1. The reaction mixture was treated during the reaction with 10μg of electrophoretically purified pancreatic DNAase (2,900 units/mg., RNAase free).

terminal addition of DNA precursor. It should be noted that the use of E. coli DNA polymerase in this system is to measure DNA template availability and is not expected to mimic mammalian DNA synthesis.

DNA in spermatozoa is almost exclusively located in the head portion. However, it was important to establish that the observed DNA template activity was confined to the sperm head fraction. Table III indicates that no significant incorporation occurs in the midpiece tail fraction.

It has been suggested by others that cytoplasmic factors may be involved in controlling nucleic acid synthesis in cell nuclei (18-21). Although specific cytoplasmic factors have not been identified as yet, some studies have suggested that specific RNA's, acidic proteins, or acidic polysaccharides might be involved (3). Recently, Kinoshita (22,23) has suggested that a heparinoid factor may be the initiator of RNA synthesis

TABLE III

Comparison of the DNA Template Activity
of Rabbit Sperm Fractions

Additions	SOURCE OF DNA TEMPLATE (equivalent to 100μg DNA/ml)			
	Sperm Heads		Sperm Midpieces-Tails	
	Control	Thiol-Treated	Control	Thiol-Treated
	[^{3}H] dTMP incorporation:cpm/hr.			
none	45	93	8	0
Heparin	199	504	3	2
Poly X	227	403	19	19
Poly C	78	121	9	0
2-Mercaptoethanol (MSH), 10mM	88	89	1	0
Heparin + MSH	2710	1710	1	3
Poly X + MSH	1120	955	27	19
Poly C + MSH	110	150	10	0

1. A complete description of the reaction mixture is given in Table I.

2. Heat denatured calf thymus DNA at a DNA concentration equal to that found in the sperm heads gave a [^{3}H]dTMP incorporation rate of 4,250 cpm/hr.

---

in the early development of sea urchin embryos. It may be of interest from the present study that heparin, among other acidic polymers, stimulates DNA synthesis in rabbit sperm nuclei. However, it is not yet known what natural factors stimulate DNA synthesis during fertilization in the rabbit.

This paper reports the first observations of the release of DNA template restrictions in isolated mammalian sperm. It is hoped that this system may provide a model for resolving the function of natural cytoplasmic factors during fertilization.

ACKNOWLEDGMENTS

The authors are grateful for the excellent technical assistance of Adele Boison, D. S. Pivec and D. C. Irby. This work was supported by NSF grant GB-41316 and a sub-contract with The Program for Applied Research on Fertility Regulation (PARFR 51), University of Minnesota.

REFERENCES

1. Monesi, V. (1971) J. Reprod. Fert., Suppl. 13, 1-14.
2. Szollosi, D. (1966) Anat. Rec. 154, 209-212.
3. Coffey, D.C., Barrack, E.R., and Heston, W.D.W. (1974) Adv. Enz. Reg. 12, 219-266.
4. Ansevin, A.T., Macdonald, K.K., Smith, C.E., and Hnilica, L.S. (1975) J. Biol. Chem. 250, 281-289.
5. Burton, K. (1968) Methods Enzymol. 12, 163-165.
6. Hutchison, W.C., Downie, E.D., and Munro, H.N. (1962) Biochim. Biophys. Acta 55, 561-570.
7. Stambaugh, R., and Buckley, J. (1969) J. Reprod. Fert. 19, 423-432.
8. Hecht, N.B. (1974) J. Reprod. Fert. 41, 345-354.
9. Blobel, G. and Potter, V.R. (1966) Science 154, 1662-1665.
10. Bollum, F.J. (1966) Procedures in Nucleic Acid Research, Eds. G.L. Cantoni, and D.R. Davies, P. 269, Harper and Row, New York.
11. Bril-Petersen, E. and Westenbrinik, H.G.K. (1963) Biochim. Biophys. Acta 76, 152-154.
12. Hendricks, D.M., and Meyer, D.T. (1965) Exp. Cell Res. 40, 402-412.
13. Coelingh, J.P., Monfoort, C.H., Rozijn, T.H., Gevers Leuven, J.A., Schiphof, R., Steyn-Parve, E.P., Braunitzer, G., Schrauk, B., and Ruhfus, A. (1972) Biochim. Biophys. Acta 285, 1-14.
14. Marushige, Y., and Marushige, K. (1974) Biochim. Biophys. Acta 340, 498-508.
15. Marushige, Y., and Marushige, K. (1975) J. Biol. Chem. 250, 39-45.
16. Calvin, H.I., and Bedford, J.M. (1971) J. Reprod. Fert., Suppl. 13, 65-75.
17. Bedford, J.M., and Calvin, H.I. (1974) J. Exp. Zool. 188, 137-156.
18. Gurdon, J.B., and Woodland, H.R. (1968) Biol. Rev. 43, 233-267.
19. Graham, C.F., Arms, K., and Gurdon, J.B. (1966) Dev. Biol. 14, 349-381.
20. Harris, H. (1970) Cell Fusion, Clarendon Press, Oxford.
21. Merriam, R.W. (1969) J. Cell Sci. 5, 333-349.
22. Kinoshita, S. (1971) Exp. Cell Res. 64, 403-411.
23. Kinoshita, S. (1974) Exp. Cell Res. 85, 31-40.