INITIATION OF DNA SYNTHESIS IN EUCARYOTES: A MODEL IN VITRO SYSTEM

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SUMMARY:

Chromatin isolated from sea urchin sperm is extremely inert as a template for highly purified E. coli DNA polymerase. Treatment of the chromatin with E. coli exonuclease III "activates" it for replication by DNA polymerases. Chromatin obtained from a wide variety of sources can be similarly "activated". The optimal magnesium requirements for the overall activation and replication reactions are closer to those reported for exonuclease III than DNA polymerase. It has not yet been possible to separate the activation and replication reactions.

In eucaryotic cells the DNA replication unit is a deoxyribonucleoprotein complex. Deoxyribonucleoprotein complexes ("chromatin") isolated from nuclei are quite inert as templates for in vitro DNA replication, using highly purified DNA polymerase. We report for the first time an in vitro system in which the DNA of chromatin may be "activated" by exonuclease III to form an efficient template for Escherichia coli DNA polymerase.

Initial studies have used chromatin prepared from sea urchin (Strongylocentrotus purpuratus) sperm. Much of the interest in the activation of sperm DNA is due to its lack of biological reactivity. S. purpuratus sperm contain no detectable DNA polymerase activity and little if any nuclease activity. The DNA of the sperm is not replicated until after the sperm enters the egg.

Chromatin was prepared by the method of Dingman and Sporn (1) and also using a modification (2) in which the extraction is carried out with 24 mM EDTA, 75 mM NaCl, pH 8.0, and the subsequent homogenization and solubilization is performed in distilled water. The E. coli DNA polymerase was obtained by

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the method of Jovin et al. (3), and is the most highly purified fraction, free of endonuclease activity. Purified exonuclease III is a by-product of the same fractionation.

Table 1. REPLICATION OF DNA OF CHROMATIN FROM SEA URCHIN SPERM AND OTHER SOURCES

Source of Chromatin and Method (ref)	A^{260} Units in Assay	μμmoles TMP^{32} incorporation per assay		Activation
		With Exonuclease III	Without Exonuclease III	
†S. purpuratus sperm (2)	0.199	184	9	20X
S. purpuratus sperm (2)	0.143	37	2	19X
S. purpuratus 200-cell embryos (2)	0.185	47	24	2.0X
Drosophila salivary glands (1)	0.147	77	11	7.0X
Mouse ascites tumor cells (1)	0.323	8.3	4.3	1.9X
Human lymphocytes (1)	0.081	57	14	4.1X

†Samples had been stored for approximately 1 year at -70°C.

The degree of activation of the chromatin template was determined by performing standard DNA polymerase assays (5) in the presence or absence of E. coli exonuclease III, using chromatin from the stated sources as a primer in the reaction. In the case of the 200-cell S. purpuratus embryos, mouse ascites tumor cells and human lymphocytes, chromatin was prepared from isolated nuclei. The reaction mixtures (0.3 ml total volume) contained: 25 μmoles Tris-maleate buffer, pH 7.8; 0.3 μmole $MgCl_2$; 0.3 μmole β-mercaptoethanol; 10 mμmoles each dATP, dCTP, dGTP and [α-^{32}P]-dTTP (about 1 to 20 x 10^4 dpm/mμmole); 0.085 μg E. coli DNA polymerase (Jovin et al. (3), Fraction 7); and 0.109 μg exonuclease III (Jovin et al. (3)). Incubation was for 30 min at 37°. TMP^{32} residues incorporated into acid-insoluble material were determined as described by Loeb (5). Non-specific adsorption of TTP^{32} without incubation was 6.7, 10.0, 10.0, 2.7, 4.2, 10.0 μμmoles dTTP, respectively, and has been subtracted from these values. The enzyme solutions used were 500-fold dilutions of the original concentrated fractions. These dilutions were made in 20 mM Tris-HCl, 20 per cent glycerol, 0.2 mM K EDTA, 1 mM reduced glutathione, 1 mg/ml BSA, final pH 7.5. 10 μl of DNA polymerase and 15 μl of exonuclease III contained the stated amounts of enzyme.

Table 1 shows that chromatin prepared from sea urchin sperm has little ability to serve as a template for the DNA polymerase. When the chromatin is treated with exonuclease III, however, the template activity is increased up to 20-fold. Exonuclease III sequentially removes nucleotides from one strand of a double-stranded DNA molecule in a 3'-5' direction; starting at a nick and leaving a 3'-OH terminus to which DNA polymerase can attach (4). In 6 preparations of sperm chromatin the increase of template activity varied from 9- to 23-fold. Table 1 also shows that chromatin isolated from a number of other tissues is relatively unreactive alone in a DNA polymerase replication system but is similarly "activated" by the action of exonuclease III.

The replication of DNA in this system is not limited to the use of DNA polymerase from E. coli. If another highly purified DNA polymerase [that from developing sea urchin embryos (5)] is used, the same phenomenon is observed - little synthesis of DNA can occur until the chromatin is "activated" by exonuclease III (Table 2). However, in this case, the components of the system are of heterologous origin.

Table 2. DNA REPLICATION OF CHROMATIN BY PURIFIED DNA POLYMERASE FROM NUCLEI OF S. FRANCISCANUS

Source of Chromatin and Method (ref)	A^{260} Units in Assay	μμmoles TMP^{32} Incorporated per Assay	
		With Exonuclease III	Without Exonuclease III
†S. purpuratus sperm (2)	0.199	39.7	4.4
S. purpuratus sperm (1)	0.116	14.8	4.6

†Samples had been stored for approximately 1 year at -70°C.

Reaction mixtures are identical to those described in the legend for Table 1 with the following exceptions: the reactions were carried out in the presence of 20 per cent glycerol and incubation was for 2 hours at 27°C. Highly purified S. franciscanus nuclear DNA polymerase (0.04 μg) (5) was used instead of E. coli DNA polymerase. Non-specific adsorption without incubation was 9.4 and 9.8 μμmoles TTP^{32} in experiments, respectively.

In other experiments we find that mild sonication and mechanical shearing do not affect the endogenous priming activity of chromatin, nor the extent to which it can be activated. DNA synthesis is proportional to the amount of chromatin present in the reaction mixture (from 0.03 to 0.27 A^{260} units) but not to the amount of exonuclease III. This implies that the number of sites on the chromatin at which the exonuclease III can bind and react is the limiting factor. It is not necessary that the chromatin be preincubated (15 min 37°) with the exonuclease III prior to replication with DNA polymerase; the level of incorporation of TTP^{32} is the same, regardless of whether preincubated or not. The optimal $MgCl_2$ concentration for the complete reaction was found to be low (0.6 mM), reflecting the optimal Mg^{++} requirement found for exonuclease III (1 mM) rather than that found for DNA polymerase (8 mM).

We have shown that chromatin, a highly stable nucleoprotein complex, can be activated for DNA replication by polymerases. We imply that chromosomal proteins, including histones, may not control the initiation and replication of DNA. Several attempts have been made to separate the process of "activation" of the chromatin by exonuclease III from the subsequent replication of DNA by the polymerase. None of these has resulted in the large degrees of activation shown when the enzymes are present and active simultaneously, even though the DNA polymerase is not at its apparent Mg^{++} optimum.

Recently Burgoyne *et al.* (6) have demonstrated a Ca^{++}-dependent factor in isolated rat liver nuclei which can render the normally inactive endogenous DNA capable of being replicated by the nuclear DNA polymerase. They suggest that sites for initiation are determined by nicking of the chromosomal DNA. Such nicks would create points of attachment for activation by exonuclease III.

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