DNA POLYMERASE ACTIVITIES IN FRACTIONATED WALKER-256 TUMOR CELL NUCLEI

N. Raghuveer Ballal, Michael S. Collins,

Richard M. Halpern and Roberts A. Smith

Departments of Chemistry and Medicine and the Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California 90024. Chemistry Department publication No. 2679.

Received August 3, 1970

SUMMARY: When isolated nuclei from Walker-256 tumor cells were fractionated into a nucleoplasmic, acidic protein and DNA-histone complex, all three fractions showed DNA synthesizing ability. The nucleoplasmic fraction was stimulated 2 to 3 fold by ATP and was most active with denatured Walker-256 DNA. The acidic protein fraction was independent of ATP and it also preferred denatured over native Walker-256 DNA. Both the nucleoplasmic and the acidic protein enzymes were active with the DNA-histone complex as promoter, but only in the presence of ATP. The DNA-histone complex was active in the presence of ATP but did not require added DNA for maximum activity.

Eukaryotic cell DNA polymerases have been characterized by their primer and template requirements. Some show a distinct preference or absolute requirement for native promoter DNA[1,2,3,4] and because of this dependence, it has been suggested that these particular polymerases are involved in the replicative process.

We have investigated the DNA-synthesizing system in isolated nuclei from Walker-256 carcinosarcoma cells and as shown for HeLa cells by Friedman and Mueller[5], the Walker-256 system is active in the absence of added DNA, but for maximal synthesis, in addition to the four deoxynucleoside-5'-triphosphates, high levels of ATP are required.

In a further extension of this work we have fractionated the nuclei essentially by the method of Wang[6] into nucleoplasmic, acidic protein and DNA-histone fractions. In this report we describe some of the properties of these three

fractions in catalyzing DNA-synthesis.

MATERIALS AND METHODS: [^{3}H]-TTP was purchased from Schwarz Bioresearch, Inc. Unlabeled deoxynucleoside 5'-triphosphates were purchased from Calbiochem. ATP was obtained from Sigma Chemical Co. Nuclei were isolated from 8-12 day old Walker-256 tumors by the method reported earlier[7]. All operations were carried out at 0-4°C. After two washes with 1 M sucrose containing 1 mM $CaCl_2$, the nuclei were pelleted by centrifugation and were extracted by homogenizing with 10 volumes of 0.14 M NaCl in 0.05 M Tris-chloride buffer (pH 7.4) containing 1 mM $MgCl_2$. After this procedure was repeated three times, the combined extracts were precipitated by adding solid ammonium sulfate to 80% saturation. The precipitate was dissolved in 30% glycerol containing 0.05 M Tris, pH 7.4 and 1 mM β-mercaptoethanol (Buffer A) and was dialyzed overnight against the same buffer. This constituted the nucleoplasmic fraction.

The extracted nuclei were then dispersed by gentle stirring for 2 hours in 100 volumes of 1 M NaCl, following which the nuclear debris was removed by centrifugation at 12,000 x g for 30 minutes. The supernatant fluid was then dialyzed against exactly 6 volumes of cold distilled water for 18 hours. This allowed the DNA histone complex to precipitate while the non-histone acidic proteins remained in solution.[6] The DNA-histone complex was collected by centrifugation and was suspended by homogenizing in buffer A. The non-histone acidic proteins were precipitated from the supernatant by adding solid ammonium sulfate to 80% saturation. The acidic protein fraction was dissolved in buffer A and dialyzed overnight against the same buffer.

Assays for DNA synthesis were performed by following the incorporation of [^{3}H]-TTP into acid-precipitable material in the presence of the other three deoxynucleoside triphosphates, using the filter paper disc technique described by Bollum[8]. The assay mixture in 0.25 ml contained Tris-CL, 6.25 μmoles pH 7.4; $MgCl_2$, 0.5 μmoles; β-mercaptoethanol, 0.25 μmoles each; ATP, 0.5 μmoles; and Walker-256 DNA, 50 μg. 50 μl of enzyme solution were added to start the reaction which was incubated at 37°. Activities are expressed in

terms of mμmoles of TMP incorporated into acid precipitable material per 30 minutes per mg of protein.

Protein was estimated by the method of Lowry et al.[9], after each fraction was dialyzed against water for 48 hours.

Walker-256 DNA was isolated by the Kay, Simmons and Dounce[10] procedure followed by a treatment with pronase and fractionation on a Sephadex (G-200) column.

RESULTS AND DISCUSSION

All three fractions when tested for their ability to catalyze DNA synthesis, were found to possess the capacity to polymerize deoxynucleoside 5'-triphosphates However, there were distinct differences in the catalytic properties of these three fractions. The nucleoplasmic fraction, as shown in Table I, revealed negligible incorporation in the absence of added DNA and maximal activity in the presence of denatured promoter DNA. Incorporation in the presence of native promoter DNA was about 30% of that with denatured DNA. ATP stimulated these activities 2 to 3-fold. The nucleoplasmic fraction was also active when the DNA-histone complex was used as the source of promoter DNA but was almost totally dependent upon ATP.

The acidic protein fraction also required added DNA for activity, and denatured DNA was preferred over native DNA (Table II). In contrast to the nucleoplasmic enzyme the acidic protein fraction was not stimulated by ATP when either native or denatured Walker-256 DNA served as promoter. In fact, addition of ATP to the reaction mixture inhibited the incorporation considerably especially in the presence of native DNA. However, ATP stimulated the incorporation of TMP about 10 fold when the DNA-histone complex was used as promoter.

The DNA-histone complex was the most active in the absence of added DNA and its activity was considerably stimulated by ATP (Table III). However, the specific activity of this fraction was only about 10% of that of either the nucleoplasmic or acidic protein fraction. The addition of native DNA to the

TABLE I

DNA POLYMERASE ACTIVITY OF THE NUCLEOPLASMIC FRACTION

Additions	TMP incorporated in 30 min	
	-ATP	+ATP
	nmoles per mg. protein	
None	0.02	0.21
DNA*, native, 50 µg	0.95	2.2
" " ($-Mg^{++}$)	--	0.16
" " (-dATP, dGTP, dCTP)	--	0.65
DNA, denatured, 50 µg (heated 100° 10', cooled)	2.57	6.67
" " ($-Mg^{++}$)	--	0.18
" " (-dATP, dGTP, dCTP)	--	1.0
DNA-Histone Complex, 50 µl	0.130	2.0
" " ($-Mg^{++}$)	--	0.18
" " (-dATP, dGTP, dCTP)	--	0.46

Assay mixture contains in µmoles per 0.25 ml: Tris-Cl (pH 7.4), 6.25; $MgCl_2$, 0.5; β-mercaptoethanol, 0.25; ATP, 0.5; and in nmoles [^{3}H]-TTP, ($2x10^5$ cpm.) 25 and dATP, dGTP and dCTP, 25 each; reaction was started by adding 50 µl of enzyme fraction.

*all DNA obtained from Walker-256 carcinosarcoma

DNA-histone complex resulted in about 30% inhibition. The activity of the DNA-histone complex was completely destroyed by treatment with either DNase I or Pronase.

All three fractions required Mg^{++} for activity. The dependence on all the four deoxynucleotides for the incorporation of [^{3}H]-TTP was different with

TABLE II

DNA POLYMERASE ACTIVITY OF THE ACIDIC PROTEIN FRACTIONS

Additions	TMP incorporated in 30 min	
	-ATP	+ATP
	nmoles per mg. protein	
None	0.38	0.93
DNA, native, 50 µg	7.60	3.90
" " ($-Mg^{++}$)	--	0.34
" " (-dATP, dGTP, dCTP)	--	1.28
DNA, denatured, 50 µg (heated 100° 10', cooled)	15.10	9.30
" " ($-Mg^{++}$)	--	0.58
" " (-dATP, dGTP, dCTP)	--	2.34
DNA-Histone Complex, 50 µl	1.05	10.50
" " (-dATP, dGTP, dCTP)	--	1.97

Conditions as in Table I.

each fraction. The nucleoplasmic and acidic protein fractions were much more dependent upon all four deoxynucleoside 5'-triphosphates than the DNA-histone complex. This result is not easily explained by the presence of terminal transferase since previous experiments have shown that this enzyme is present in the cytoplasmic fraction and absent in the nucleus[7]. Recently it has been shown that a large proportion of [^{3}H]-thymidine added to HeLa cells in culture was converted to [^{3}H]-TTP which was not easily dissociated from the HeLa DNA even after exhaustive dialysis.[11] Thus a pool of deoxynucleoside-5'-triphosphates bound to DNA is suggested, but in our system preincubation of the DNA-histone complex with ATP and Mg^{++} did not render it more dependent

TABLE III

DNA POLYMERASE ACTIVITIES OF THE DNA-HISTONE COMPLEX

Additions	TMP incorporated in 30 min	
	-ATP	+ATP
	nmoles per mg. protein	
None	0.23	0.73
" (-Mg^{++})	--	0.18
" (-dATP, dGTP, dCTP)	--	0.42
DNA'ase I, 10 µg	--	< 0.01
Pronase, 0.50 mg	--	< 0.01
DNA, native, 50 µg	0.36	0.46
" " (-Mg^{++})	--	0.10
" " (-dATP, dGTP, dCTP)	--	0.22
DNA, denatured, 50 µg (100° for 10' cooled)	0.36	0.65
" " (-Mg^{++})	--	0.17
" " (-dATP, dGTP, dCTP)	--	0.47

Assay conditions as in Table I.

on all four deoxynucleotides. However, when the DNA-histone complex was used as promoter DNA with either the nucleoplasmic or acidic protein fractions (Tables I and II) dependence on all four deoxynucleotides was much more apparent One possibility to explain these results is that in the DNA-histone complex, polymerase molecules may be limited and cannot overcome the pool of bound deoxynucleotides.

Wang[3] has studied a DNA polymerase from the acidic protein fraction of Walker-256 tumor. This enzyme was almost absolutely dependent upon native

calf thymus DNA for activity. However, the acidic protein fraction obtained by us showed a preference for heat denatured rather than native Walker-256 DNA. Wang's acidic protein enzyme showed only slight activity (14%) when reconstituted with the DNA-histone complex. We also observed this slight activity when the acidic protein fraction was assayed with the DNA-histone complex in the absence of ATP. But in the presence of ATP, the activity was about 60% of that with denatured DNA, and about 160% of that with native Walker-256 DNA (Table II).

The polymerase activity associated with the DNA-histone complex, even though considerably lower than the other two fractions, is significant in that it requires no added promoter DNA. It is possible that the DNA-histone complex may have to be modified by a reaction involving ATP before it can serve as template. This view is supported by the fact that the acidic protein fraction which is ATP independent, is active with DNA-histone complex only in the presence of ATP. Since both the DNA histone complex and the nucleoplasmic fraction are stimulated by the addition of ATP to the reaction mixture, we do not know whether the ATP stimulation in both these fractions is caused by the same factor(s).

Recently, Smith, Schaller and Bonhoeffer[12] demonstrated a membrane associated DNA-replicating system from the DNA polymerase-deficient mutant of *E. coli*. Their system was also stimulated by ATP and apparently elaborated DNA semiconservatively. Experiments are in progress with the Walker-256 system to investigate more thoroughly the product formed.

ACKNOWLEDGMENT: This investigation was supported by grants from the United States Public Health Service (GM 13407) and The Julius and Dorothy Fried Research Foundation.

REFERENCES:

1. Loeb, L. A., J. Biol. Chem. 244, 1672 (1969).
2. Chang, L. M. S. and Hodes, M. E., J. Biol. Chem. 243, 5537 (1968).
3. Wang, T. Y., Proc. Soc. Exptl. Biol. Med. 129, 469 (1968).
4. Howk, R. and Wang, T. Y., Arch. Biochem. Biophys. 133, 238 (1969).
5. Friedman, D. L., and Mueller, G. C., Biochim. Biophys. Acta 161, 455 (1968).

6. Wang, T. Y., J. Biol. Chem. 242, 1220 (1967).
7. Rothschild, M. L., Halpern, R. M. and Smith, R. A., Biochim. Biophys. Acta 169, 218 (1968).
8. Bollum, F. J. in Procedures in Nucleic Acid Research (G. L. Cantoni and D. R. Davies, Eds.), Harper and Row, New York, p. 296 (1966).
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem. 193, 265 (1951).
10. Kay, E. R. M., Simmons, N. S. and Dounce, A. L., J. Am. Chem. Soc. 74, 1724 (1952).
11. Habener, J. F., Bynum, B. S. and Schack, J., J. Mol. Biol. 49, 157 (1970).
12. Smith, D. W., Schaller, H. E. and Bonhoeffer, F. J., Nature 226, 711 (1970).