DNA-DEPENDENT DNA POLYMERASES FROM HELA CELL NUCLEI
II. TEMPLATE AND SUBSTRATE UTILIZATION

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Summary: Two separable DNA polymerases which have been partially purified from the nuclei of HeLa cells prefer as primer a double-stranded DNA into which 3'-hydroxyl termini have been introduced by nuclease action. These enzymes differ in their ability to use synthetic polydeoxynucleotides as templates. Neither of the enzymes can utilize a polyribonucleotide strand as a template. Thus the template response of the HeLa DNA polymerases differs from that reported for the RNA-dependent DNA polymerases of the RNA tumor viruses. The DNA-dependent HeLa DNA polymerases also show a limited ability to polymerize single deoxynucleotides.

Introduction

Nuclei of HeLa cells contain two well-differentiated DNA polymerase activities designated nuclear I and nuclear II (1). A single DNA polymerase activity found in the cytoplasm very closely resembles nuclear enzyme II and may be identical to it (1). In order to further examine the relationship between the two nuclear enzymes and the cytoplasmic DNA polymerase, we have studied the primer and template activities of natural and modified DNA's and of synthetic polymers with these enzymes. The nuclear I and nuclear II enzymes are found to differ from one another in their relative abilities to utilize various synthetic polydeoxynucleotide templates. DNA polymerase obtained from the cytoplasm behaves very much like nuclear enzyme II in its response to the various templates tested.

Materials and Methods

Materials: Purified calf thymus DNA polymerase and deoxynucleotidyl terminal transferase were supplied by Dr. A. Ramel, and the polydeoxynucleotides, $(pT)_9$, poly dT, and dG:dC were provided by Dr. A. Nussbaum, both of the Chemical Research Division of Hoffmann-La Roche Inc. Poly rA was obtained

from Miles Laboratories, Elkhart, Indiana. The synthetic templates*, dAT, rG:dC, dT:rA, and rA:rU were a gift from Dr. S. Spiegelman. The partial purification of DNA polymerase activities from HeLa cells was done as previously described (1).

Treatment of DNA with Pancreatic DNase: Native salmon sperm DNA (2.5 mg per ml in 0.01 M Tris, pH 7.4 containing 5×10^{-3} M $MgCl_2$) was incubated with pancreatic DNase (40 ng of DNase per mg of DNA) at 37° for 25 min. DNase was then inactivated by heating for 7 to 10 min at 60°. Double-stranded DNA treated in this manner is rendered 25% acid soluble and is maximally activated in its priming ability for the HeLa cell DNA polymerases.

Results

I. Primer and Template Requirements: The HeLa cell DNA polymerases described here are dependent upon the presence of DNA as primer and show no activity in the absence of added template. The products of such synthesis are found to be completely (>98%) solubilized by pancreatic deoxyribonuclease and are not degraded by ribonuclease. The DNA requirement cannot be substituted for by RNA since none of the HeLa cell DNA polymerase can utilize RNA as template.

Table 1 shows that the best DNA primer for each of the DNA polymerases is nicked native DNA, i.e., DNA in which 3'-hydroxyl termini have been introduced by the action of pancreatic deoxyribonuclease (see Methods). Such nicked native DNA is much more active as a primer than is either native or denatured DNA. Heat denaturation of nicked native DNA renders it at least 50% less effective as a primer. This indicates that in addition to a requirement for 3'-hydroxyl termini, the enzymes need some double-stranded structure for optimal priming activity. Similar results are found with either salmon sperm or HeLa DNA. The purified HeLa DNA polymerases utilize native or denatured DNA at a very low rate relative to an activated DNA.

*dAT = the double-stranded, alternating copolymer composed of dA_p and dT_p units. dG:dC, rG:dC, dT:rA, and rA:rU are all duplexes of the homopolymers indicated. polydeoxyguanylate (dG), polydeoxycytidylate (dC), polyriboguanylate (rG), polydeoxythymidylate (dT), polyriboadenylate (rA), and polyuridylate (rU). dNTP = deoxynucleoside triphosphate.

TABLE 1

UTILIZATION OF NATURAL TEMPLATES

BY HELA DNA POLYMERASES

DNA [1]	ENZYME					
	Nuclear I		Nuclear II		Cytoplasm	
	pmoles[4]	%	pmoles[4]	%	pmoles[4]	%
Nicked Native [2]	220	100	666	100	828	100
Nicked Native [3] (denatured)	36	16	236	35	360	44
Native	26	12	28	4	2	<1
Denatured	0	0	3	<1	3	<1

[1] Salmon sperm DNA was utilized as the template in these studies.

[2] The preparation of nicked native DNA by treatment with pancreatic deoxyribonuclease is described in Methods.

[3] The same nuclease-treated DNA used above, denatured by heating to 100° for 30 min followed by rapid cooling in an ice bath.

[4] Total picomoles of nucleotide incorporated.

The DNA polymerase assay measures the incorporation of ^{3}H-deoxynucleoside triphosphates into acid insoluble material. Each assay contained: bovine serum albumin, Fraction V, 90 µg; Tris-HCl buffer (pH 8.5), 10 µmoles; $MgCl_2$, 1.5 µmoles; Ditliothreitol, 0.1 µmole; DNA, 100 µg; ^{3}H-deoxyadenosine triphosphate (dATP), 20 nmoles (5 x 10^7 cpm per µmole); unlabeled deoxythymidine triphosphate (TTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 20 nmoles of each; 25-40 µliters of enzyme solution in a final volume of 200 µliters. The enzyme fractions employed were Sephadex G-200 fractions which had been purified from 150-500 times (1). Enzyme proteins added to the appropriate assays were: Nuclear I, 0.72 µg per assay; nuclear II, 0.45 µg per assay; and cytoplasmic enzyme, 0.63 µg per assay. Assays were incubated at 37° for 30 min. Acid precipitation of the reaction products and preparation for counting were done as previously described (2) except that the scintillation counting fluor consisted of 4.5 g of 2,5-diphenyloxazole, 100 mg of 1,4-Bis(2-(4-methyl-5-phenyloxazoly))-benzene, and 2.0 ml of glacial acetic acid per liter of toluene.

Pretreatment of salmon sperm DNA with micrococcal DNase, which is known to introduce 3'-phosphoryl termini into the DNA, causes a 7-fold decrease in the priming ability of the DNA.

II. Utilization of Synthetic Templates: The ability of HeLa cell DNA polymerases to utilize a number of synthetic templates was studied (Table 2).

TABLE 2

UTILIZATION OF SYNTHETIC TEMPLATES

BY HELA DNA POLYMERASES

TEMPLATE	ENZYME					
	Nuclear I		Nuclear II		Cytoplasm	
	pmoles*	%	pmoles*	%	pmoles*	%
Nicked Native DNA	519	100	116	100	342	100
dAT	338	65	19	16	91	27
dT:rA	30	6	<1	<1	1	<1
dG:dC	157	30	76	6	23	7
rG:dC	1	<1	1	1	0	0
rA:rU	0	0	1	<1	2	<1
$(pT)_9$	3	<1	0	0	0	0
$(pT)_9$:rA	2	<1	2	2	<1	<1
poly dT	1	<1	1	1	<1	<1

* picomoles of substrate incorporated are calculated on the basis of the polymerization of all 4 deoxynucleotides when DNA is the template and on the basis of polymerization of dAMP and TMP when dAT is used as template.

Assays were performed as described in Table 1 except that each assay contained: 2.0 µg of template instead of 100 µg; assays with nuclear enzyme II and cytoplasmic enzyme were performed at pH 6.5 (optimum for these two enzymes at low template levels, ref. 1); each assay contained deoxynucleoside triphosphate substrates appropriate for that assay, i.e., with nicked native DNA, ^{3}H-dATP plus the other three deoxynucleotides unlabeled; with dAT, ^{3}H-dATP and TTP; with dT:rA, rA:rU, $(pT)_9$:rA, $(pT)_9$, and poly dT, ^{3}H-dATP; with dG:dC and rG:dC, ^{3}H-dGTP. Each nucleoside triphosphate was added at 20 nmoles per assay and the labeled compounds has specific activities of 5×10^7 cpm per µmole. Enzyme proteins added to the appropriate assays were: Nuclear enzyme I, 1.8 µg per assay; nuclear enzyme II, 1.8 µg per assay; and cytoplasmic enzyme, 2.1 µg per assay.

Regardless of the labeled nucleoside triphosphate used as a substrate no significant activity was seen with rA:rU, rG:dC, $(pT)_9$, $(pT)_9$:rA or poly dT. However, dAT and dG:dC showed significant template activity with each of the HeLa polymerases when compared to nicked native salmon sperm DNA. With these two templates nuclear enzyme I shows a 2 to 4-fold higher rate of synthesis

than does nuclear enzyme II or the cytoplasmic enzyme. In all cases, the dC strand of dG:dC is preferentially copied with the subsequent incorporation of dGMP. The incorporation of dCMP with the dG:dC template is only 10% that of dGMP. In contrast, as noted above, rG:dC has little template activity for the HeLa DNA polymerases so that the substitution of ribo G for deoxy G strongly affects the ability of the enzymes to copy the complementary dC strand. dAT shows higher template activity than any other synthetic template with any of the HeLa DNA polymerases described here (Table 2). However, dT:rA shows significant template activity only with nuclear enzyme I. Using this template one observes predominantly the copying of the dT strand with the resulting incorporation of dAMP into the acid-insoluble product. The incorporation of TMP, possibly resulting from the reading of the rA strand, occurs at a rate only about 10% that of dAMP. The failure of the HeLa DNA polymerases to use single-stranded DNA (Table 1) as a template is also reflected in their inability to use poly dT.

III. Incorporation of Single Deoxynucleotides: The data in Table 3 show the polymerization of single ^{3}H-dNTP in the presence and absence of the other three dNTPs. Table 3 illustrates the phenomenon previously reported by Bollum (3) who showed that the rate of incorporation of a single dNTP by calf thymus deoxynucleotidyl terminal transferase is inhibited in the presence of the other dNTPs. This is the opposite effect seen with the calf thymus DNA polymerase which shows a marked stimulation of nucleotide incorporation when all 4 dNTPs are present (Table 3). The HeLa DNA polymerases clearly are similar to the calf thymus DNA polymerase, and other DNA polymerases, in that the maximal rate of incorporation of a single dNTP requires the presence of the other three dNTPs. Table 3 shows this to be true irrespective of which ^{3}H-dNTP is used to measure incorporation. Nevertheless, the ability of the HeLa DNA polymerases to polymerize a single dNTP, in the absence of any others, is apparent. This characteristic is particularly significant with nuclear enzyme I. It should be noted in Table 3 that the actual total amount of dNTP converted to polymeric form would be about 4 times greater when all four dNTPs

TABLE 3

INCORPORATION OF ^{3}HdATP IN THE PRESENCE AND

ABSENCE OF ALL FOUR DEOXYNUCLEOSIDE TRIPHOSPHATES

ENZYME	SUBSTRATE							
	^{3}HdATP	^{3}HdATP dCTP dGTP dTTP	^{3}HdTTP	^{3}HdTTP dATP dGTP dCTP	^{3}HdGTP	^{3}HdGTP dCTP dATP dTTP	^{3}HdCTP	^{3}HdCTP dGTP dATP dTTP
	pmoles of ^{3}H-labeled substrate incorporated							
HeLa Nuclear I	19	42	51	61	35	53	41	48
HeLa Nuclear II	19	164	30	185	43	193	26	119
HeLa Cytoplasm	8	84	*	*	*	*	*	*
Calf Thymus DNA Polymerase	8	88	*	*	*	*	*	*
Calf Thymus Terminal Transferase	260	120	*	*	*	*	*	*

Each assay contained imidazole buffer (pH 7.5), 10 µmoles; $MgCl_2$, 1.0 µmole; dithiothreitol, 0.1 µmole; nicked native salmon sperm DNA, 100 µg; deoxynucleotides as indicated, 20 nmoles of each (tritiated deoxynucleotides have an activity of 5×10^7 cpm per µmole); and 25-50 µl of enzyme solution in a final volume of 200 µliters. Enzyme proteins added to the appropriate assays were: HeLa nuclear I, 0.72 µg; HeLa nuclear II, 0.45 µg; HeLa cytoplasmic enzyme, 0.63 µg; calf thymus DNA polymerase, 20 µg; calf thymus deoxynucleotidyl terminal transferase, 1.8 µg.

The values shown represent pmoles of only the ^{3}H-labeled deoxynucleoside triphosphate incorporated.

*not done

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are present in reactions catalyzed by the DNA polymerase. The HeLa DNA polymerases also differ from the calf thymus terminal transferase in their failure to use pure oligodeoxynucleotides such as $(pT)_3$ or $(pT)_9$ as primers (7).

Discussion

The presence of 3'-hydroxyl termini for maximal activity of DNA polymerases *in vitro* seems to be a general phenomenon (4,5) and is also true for the HeLa DNA polymerases. The treatment of native DNA with pancreatic deoxyribonuclease I, to introduce 3'-hydroxyl ends, greatly increases the ability of this DNA to serve as primer, whereas the introduction of 3'-phosphoryl ends into DNA

decreases its priming ability for the HeLa DNA polymerases.

Since HeLa is a cell of malignant origin it was important to compare these HeLa DNA polymerases with the virion associated RNA-dependent DNA polymerases of the RNA tumor viruses. The HeLa DNA polymerases described here are unlike the virion polymerases since RNA does not serve as template for any of the HeLa enzymes. The virion DNA polymerase of avian myeloblastasis virus (AMV) reported by Spiegelman et al. (6) copies the ribo strand of dT:rA while the HeLa enzymes were noted to be preferentially copying the deoxy strand. Both the HeLa and the virion enzymes utilize dG:dC as a template by copying the dC strand with the incorporation of dGMP. However, a striking difference is observed when rG:dC is provided as template. The HeLa cell enzymes are unable to use this primer-template even though the dC strand, which was previously copied in dG:dC, is still present. The AMV enzyme, on the other hand, shows a faster rate of incorporation of dGMP when rG:dC is used as primer. Therefore, the HeLa enzymes described here seem to have a preference for a deoxy structure in both strands of the primer-template molecule. We have not, however, ruled out the possibility that an RNA-dependent DNA polymerase of the type found in oncogenic RNA viruses may exist in HeLa cells.

References

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