DNA POLYMERASE FROM HELA CELL NUCLEI: LEVELS OF ACTIVITY DURING A SYNCHRONIZED CELL CYCLE[1]

Daniel L. Friedman

Department of Molecular Biology
Vanderbilt University, Nashville, Tennessee 37203

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Summary

HeLa cell nuclei prepared from aqueous media contained 25-30% of the total polymerase of the cell in a form which was not released by repeated washing of the nuclei. Cell cycle studies revealed that the nuclear polymerase activity increased 3-5 fold as cells approached the DNA synthetic phase, remained high until completion of DNA synthesis and then declined. In contrast, the cytosol polymerase activity remained relatively constant throughout the cell cycle.

Mammalian cell DNA polymerase is found largely in the cytosol when cells are lysed in aqueous media[1-4]; however when non-aqueous extraction methods are used, the enzyme is distributed about equally between nucleus and cytoplasm[5,6]. Although the relationship between these enzymes is unclear, studies with an aqueous nuclear system from HeLa cells suggested that the cytosol enzyme did not play a role in nuclear DNA synthesis. A requirement for other cytosol proteins was, however, demonstrated[7].

The present studies with HeLa cells describe a nuclear DNA polymerase which, though largely soluble within the nucleus, is not lost during aqueous extraction. Levels of this activity are determined at various times in a synchronized cell cycle and the pattern is compared to that for the cytoplasmic DNA polymerase. The results demonstrate that as cells proceed toward S-phase, the DNA polymerase levels in isolated nuclei increase markedly, with little change in the cytosol enzyme. The findings are consistent with a role for the nuclear enzyme in DNA replication and would appear to reopen the possibility that polymerase levels might be involved in triggering DNA replication.

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Methods

The strain of HeLa cells used, the standard procedure for its culture in suspension and in monolayer, and the synchronization methods have been previously described[7,8]. To synchronize cells, a thymidineless state was induced by addition of amethopterin (final concentration 10^{-6} M) and adenosine (5×10^{-5} M). During the 16 hours with amethopterin, cells progress through interphase and become competent to replicate DNA. Addition of thymidine leads to a synchronous wave of DNA synthesis. The use of other synchronizing agents is described in the figures.

The method for preparing nuclei, using a Dounce homogenizer, was as previously described[7]. Nuclei were washed by sedimenting at 300 x g for 3 minutes and resuspending to a concentration of 50×10^6 nuclei per ml with a solution that contained 15 mg/ml bovine serum albumin (Calbiochem, B grade), 0.002 M $MgCl_2$, and 0.01 M KH_2PO_4-K_2HPO_4 buffer (pH 7.7). With 3 washes, this procedure led to a 60-80% yield of nuclei, whereas in the absence of bovine serum albumin the yield was only 30-50%. The nuclei were free of other particulate material and lacked cytoplasmic tags when observed by phase contrast microscopy.

DNA polymerase was assayed essentially by the disc method of Bollum[9]. The reaction mixture contained the following components in a final volume of 0.12 ml: cell extract (50×10^6 cells/ml), 0.04 ml; $MgCl_2$, 1.2 µmoles; Tris-HCl buffer, pH 8.0, 3.6 µmoles; pretreated DNA, 28.8 µg; dATP, dGTP, dCTP, 0.12 µmoles each; ATP, 0.6 µmoles; H^3TTP, 0.06 µmoles (16µc/µmole). The DNA was heat denatured and treated for 6 minutes at 37° with one µg/ml of pancreatic DNAase (Worthington, 2400 units/mg) in a reaction mixture containing $MgCl_2$ (10 mM) and Tris-HCl (80 mM, pH 8.0). The mixture was then heated in a boiling water bath for 10 minutes.

Results

Cultures blocked for 16 hours with amethopterin were reversed for 3 hours with thymidine, leading to synchronization of 70% of the cells in mid S-phase.

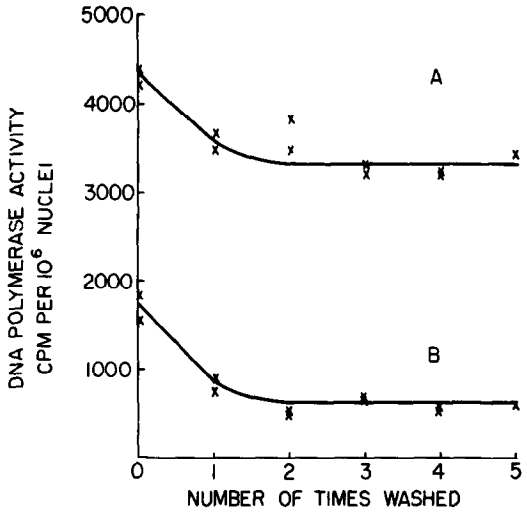

Figure 1 Effect of washing of nuclei on levels of DNA polymerase activity. Curve A: Suspension cultures (2.6 X 10^5 cells/ml) were treated with amethopterin and adenosine for 16 hours and reversed for 3 hours with thymidine. Nuclei were prepared as described in Methods and assayed directly. Nuclei were counted in a hemocytometer after each wash. The final yield of nuclei, after 5 washes, was 54.8%. Curve B: Nuclei were prepared from randomly growing cells (3.6 X 10^5 cells/ml). The final yield was 47.4%.

Nuclei were isolated from these cells, washed repeatedly with an albumin solution (see Methods) and assayed for DNA polymerase activity. The results are shown in Figure 1 (curve A). The amount of DNA polymerase per nucleus in these preparations decreased during the first 2 washes; however no further loss occurred during the subsequent 3 washes. A similar curve was observed with nuclei from randomly growing cells (curve B). In both cases the amount of activity lost in the early washes approximated the expected contamination by cytoplasmic DNA polymerase, on the assumption that 10% of the cytoplasm was carried over in the initial sedimentation. Thus it appeared that the DNA polymerase in these nuclei was not lost during extraction. The amount of polymerase in washed nuclei from S-phase cells accounted for about 25-30% of the total polymerase of the cell. The levels of activity were similar whether measured in whole nuclei or nuclear lysates. When nuclei were ruptured by either sonic oscillation or in a blender, between 60% and 80% of the activity was soluble as judged by sedimenting for 1 hour at 100,000 x g.

The DNA polymerase from washed, sonicated nuclei from S-phase cells was partially purified by ammonium sulfate fractionation (see legend of Table II for method), and some of its properties were studied. There was an absolute requirement for DNA, and like the cytoplasmic polymerase[7], the nuclear enzyme showed a strong preference (12 fold) for denatured DNA as template as compared with native DNA. Requirements for all four deoxyribonucleotide triphosphates and Mg^{++} were also observed. In the absence of the 3 unlabled triphosphates the incorporation was reduced from 1250 cpm to 80 cpm. The reaction was stimulated by a controlled pretreatment of the DNA template with pancreatic DNAase[10]. Pretreatment with micrococcal nuclease or sonication did not stimulate incorporation. In all of the present studies denatured DNA which was pretreated (see Methods) with pancreatic DNAase was used in the assay.

Table I. Nuclear DNA polymerase activity in random and S-phase cells.

Experiment No.	Preparation	DNA Polymerase Activity
		cpm/0.02 ml extract
1	Nuclei from random cells	566
	Nuclei from S-phase cells	2440
	Nuclear sonicates from random cells	582
	Nuclear sonicates from S-phase cells	2029
2,3	Ammonium sulfate fraction of nuclei from random cells	136, 147
	Ammonium sulfate fraction of nuclei from S-phase cells	824, 723

Cells were grown in suspension cultures. Synchronized cells (2.5×10^5 cells/ml) were prepared by treating with amethopterin and adenosine for 16 hours and with thymidine (5 $\mu g/10^6$ cells) for 4 hours. The population of random cells was 2.95×10^5 cells/ml at the time of harvesting. Nuclei were washed 2 times and assayed directly or sonicated (15 seconds, 1.5 amperes on a MSE sonic oscillator) prior to assay. In Experiments 2 and 3 saturated $(NH_4)_2 SO_4$ (pH 7.6, containing 0.0005 M EDTA and 0.01 M mercaptoethenol) was added to nuclear sonicates to bring the concentration to 30% saturation. After 30 min. the mixture was sedimented (30,000 x g for 20 min.) and $(NH_4)_2SO_4$ was added to the supernatant to 50% saturation. The mixture was sedimented and the precipitate taken up in 0.05 M Tris pH 7.7 containing 0.0005 M EDTA, and 0.01 M mercaptoethenol, and dialyzed overnight against the same buffer.

The levels of nuclear activity were compared in cells in mid S-phase with levels in randomly growing cells, using several different preparations (Table I). S-phase nuclei were found to contain 3-5 times more activity than nuclei from randomly growing cells whether activities were measured in intact nuclei, in nuclear sonicates, or in ammonium sulfate fractions. This latter finding would tend to rule out the possibility that the differences were due to a soluble factor. That the difference was not an artifact of amethopterin treatment was shown by the similar findings obtained when hydroxyurea or high levels of thymidine were used to block DNA synthesis (Table II). In these experiments cells were brought to the beginning of S-phase without reversing the blockade, indicating that DNA synthesis is not required for the increase

Table II. Effect of hydroxyurea, amethopterin and high levels of thymidine upon nuclear activity.

Experiment No.	Treatment	Nuclear DNA Polymerase Activity
		cpm/10^6 nuclei
1	Random cells	744
	Hydroxyurea - 16 hours	2030
	High levels TdR - 16 hours	2120
	Amethopterin - 16 hours	1460
2	Random cells	702
	Amethopterin - 16 hours	2470
3	High levels TdR - 16 hours	2568
	High levels TdR - 16 hours washed and incubated 15 hours	610

In Experiment 1 cells were randomly grown in suspension or were blocked for 16 hours with hydroxyurea (10^{-3} M), high levels of thymidine (TdR) (10^{-3} M) or with amethopterin. The inhibitions were not reversed. Nuclei were prepared and washed 2 times, suspended to 50 X 10^6/ml and assayed. In Experiment 2, the nuclei were prepared by the method of Fisher and Harris[11] and washed 2 times as described in Methods. In Experiment 3, cells were grown in monolayer culture. Four bottles were treated with high levels of TdR. After 16 hours, 2 bottles were harvested and pooled and nuclei prepared. The media in the other two bottles was decanted, replaced with fresh media and the bottles were incubated for 15 hours. During this time the cells proceeded through S-phase, G-2 phase and mitosis, to the next G-1 phase. Cells were then harvested, and the nuclei were prepared and assayed.

in polymerase activity in isolated nuclei.

The possibility that the activity of S-phase nuclei was due to contamination of nuclear preparations by whole cells was considered since there were up to 5% whole cells (5 whole cells for every 95 nuclei) in these preparations. This appeared unlikely since there was no significant difference in the percentage of whole cells between preparations from synchronous S-phase cells and those from randomly growing cultures. To test this possi-

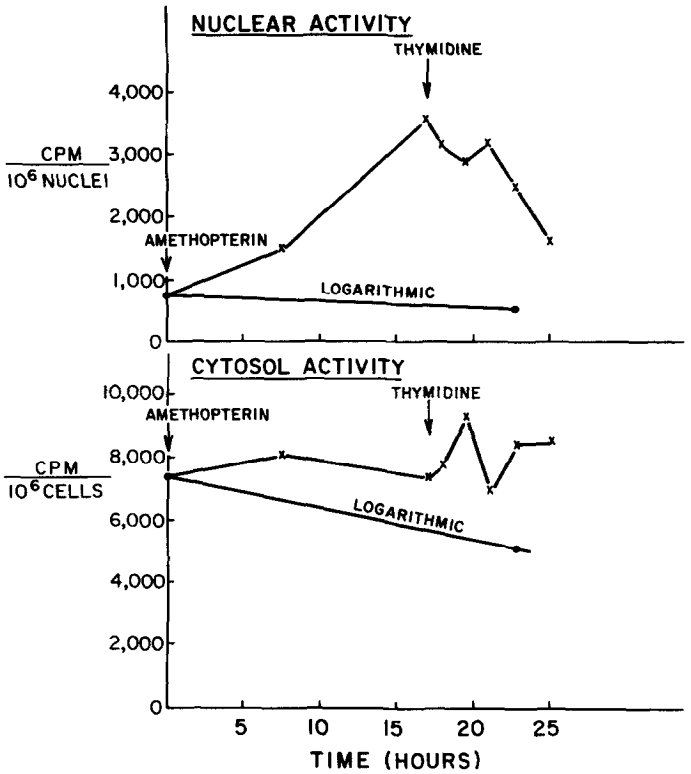

Figure 2 Levels of nuclear and cytosol DNA polymerase activity during the cell cycle. Cells were grown in 2000 ml Florence flasks. Synchronization was carried out with amethopterin and adenosine (arrow) and reversal with thymidine (arrow) as described in Methods. Other cells were grown randomly (logarithmic). As predicted, the cell count increased from 2.1 X 10^5/ml to 2.4 X 10^5/ml after 17 hours with amethepterin and did not change thereafter. The logarithmic cells increased from 2.0 X 10^5 to 3.7 X 10^5 during the experiment. Aliquots of 300 ml were removed at the indicated intervals. After preparing lysates, the nuclei were sedimented and the supernatant decanted and centrifuged at 40,000 x g for 45 min. The supernatant is designated cytosol. The nuclei were washed 3 times, suspended in 0.05 M Tris-HCl pH 7.7 and both nuclei and cytosol fractions were frozen at -70°. Later, samples were thawed rapidly and assayed.

bility nuclei were isolated with less than 1% whole cell contamination by the method of Fisher and Harris[11], which utilizes detergent and prolonged shearing of cells. The results (Experiment 2, Table II) were essentially the same as those obtained in the previous experiments, rendering unlikely the possibility that whole cells were producing the observed differences.

These experiments suggested that polymerase activity increased in isolated nuclei as cells prepared for DNA synthesis. More complete cyclic patterns of

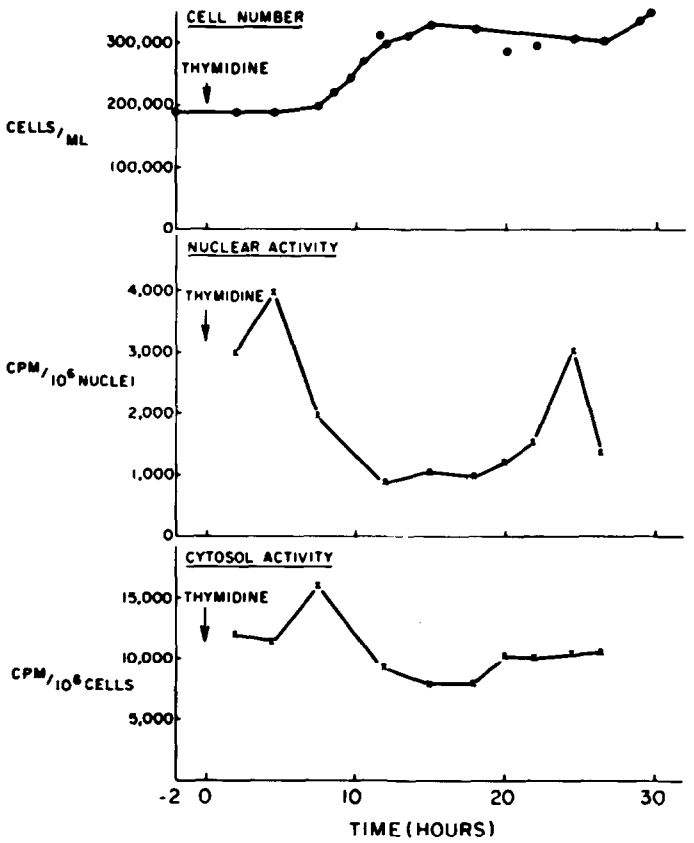

Figure 3 Levels of nuclear and cytosol DNA polymerase activity during the cell cycle. The methods are similar to those in Figure 2. Amethopterin and adenosine were added 16 hours before zero time. Thymidine (10 μg/10^6 cells) was added at zero time and additional thymidine (10 μg/10^6 cells) was added at 18 hours. A second dose of adenosine was added at 8 hours. The upper graph shows the cell number as determined on a Coulter cell counter. In addition to the determinations of nuclear and cytosol polymerase activity with H^3TTP, DNA synthesis in intact cells was monitored between 19 and 26 hrs. by addition of H^3TdR to an aliquot of cells. The rate of DNA synthesis in the whole cells increased 3-fold between 19 and 23 hours, exhibited a peak between 23 and 25 hours, and then began to decrease.

distribution of nuclear and cytosol enzymes are shown in Figures 2 and 3. In contrast to the nuclear activity, the cytosol activity did not undergo any large percentage changes during the amethopterin block or after its reversal (Fig. 2). The nuclear activity increased during the block, remained high for 6 hours, the length of S- phase, and then began to decrease. Figure 3 begins with the reversal of the amethopterin block and shows the changes in activity in the subsequent cell cycle. The results demonstrate that the nuclear DNA polymerase level rises during the subsequent S-phase without further addition of blocking agents, indicating that the change occurs spontaneously. The increase in cytosol activity observed at 8 hours could have been due to the passage of the nuclear enzyme to the cytosol; however, this effect was not observed in all experiments. The fall in cytosol activity at 12 hours resulted from cell division.

Discussion

Previous studies[12,13,14] on the levels of DNA polymerase in continuous cultures from mammalian cells have indicated that there are no large percentage changes in activity during the cell cycle. It has been inferred from this that the level of this enzyme does not play a role in initiating DNA replication. However, the distribution of enzyme between nucleus and cytoplasm was not studied in detail. Gold and Helleiner[12] and Littlefield *et al*[13] did present evidence suggesting a small redistribution of enzyme from cytoplasm to nucleus in L-cells after reversal of a DNA synthesis blockade. However, neither of these studies dealt with changes in nuclear activity as cells approached DNA synthesis.

The results of the present paper suggest that nuclei accumulate DNA polymerase activity as cells prepare for DNA replication, a temporal pattern similar to that previously reported for thymidine kinase levels in whole cells[15]. The levels remain high during S-phase and then decline. This is in contrast to levels of the cytosol enzyme which remain relatively constant during the cell cycle. An alternative interpretation, that there is a change

in permeability of the nuclear membrane during the S-phase such that the polymerase cannot leak out during isolation, seems less likely. This explanation would require that the membrane permeability change back at the completion of DNA synthesis. The data in Figure 1 argues against this possibility since the loss in activity during washing is about the same in log and S-phase cells, in both cases an amount which could easily have been due to cytoplasmic contamination.

These findings reopen the question of whether DNA polymerase levels could play a role in controlling the initiation of DNA replication in the nucleus. It becomes of interest also to determine whether the nuclear polymerase is identical with the cytoplasmic enzyme. Studies are in progress on this question and a preliminary report suggesting a kinetic difference between nuclear and cytoplasmic preparations has been presented[16]. It is not yet clear however whether or not this represents a difference between the two enzymes themselves or some other difference in the preparations.

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