Articles

Binding of the DNA Polymerase α–DNA Primase Complex to the Nuclear Matrix in HeLa Cells†

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Received February 24, 1987; Revised Manuscript Received May 12, 1987

ABSTRACT: It is well-known that there are multiple forms of DNA polymerase α. In order to determine which form(s) is (are) tightly bound, the activities were dissociated from DNA-poor nuclear matrices, with octyl β-D-glucoside. Sucrose gradient sedimentation analysis revealed three bands with *s* values of 7.5, 10.5, and 13. The 7.5S form was free of DNA primase and represented only 10% of the total DNA polymerase α bound to the nuclear matrix. The 13S and the 10.5S forms each contained DNA primase activity. The 10.5S form comprised 85% of the DNA polymerase α activity and 95% of the DNA primase activity, dissociated from the nuclear matrix. Neither temperature of nuclease digestion nor various salt treatments of nuclei had significant effects on the proportions of DNA polymerase α and DNA primase activities bound to, or subsequently dissociated from, nuclear matrices. In a comparison of primase activity bound to the nuclear matrix, dissociated from the nuclear matrix, and in the soluble fraction, it was found that the bound activity had a lower ATP dependence, had less KCl inhibition, and was less sensitive to heat, compared to the dissociated and soluble activities. No differences in Mg^{2+} or pH dependence were noted. The amounts of DNA polymerase α and DNA primase activities bound to the nuclear matrix varied over the cell cycle of synchronized cells. Over the S phase, there were two peaks of matrix-bound DNA primase and two peaks of subsequently dissociated DNA polymerase α–DNA primase complex. Each of these two peaks of enzyme activity strongly correlated with the two bursts of DNA synthesis over S phase, suggesting that biphasic binding of the DNA polymerase α–DNA primase complex to the nuclear matrix is responsible for the biphasic nature of DNA synthesis in HeLa. Following the two peaks of activity during S phase, the amount of matrix-bound DNA polymerase α–DNA primase complex dropped to relatively low levels through G_2M and remained low until late G_1, when there was a burst of DNA polymerase α, but not of DNA primase, activity. Just before reentry into S phase, the activity of the matrix-bound DNA polymerase α–DNA primase complex increased again, suggesting that the dynamic binding of this complex to the nuclear matrix may be one of the factors that determines the onset of DNA synthesis (i.e., S phase).

In the simplest procaryotic DNA replication system, a small phage or plasmid with an oriC sequence, more than 20 different proteins must function coordinately (Kornberg, 1980, 1982). Hence, it would seem reasonable that at least this many proteins are involved in mammalian DNA synthesis. The pursuit of the elusive mammalian replisome has finally begun to show promise with the finding that DNA primase exists tightly associated with DNA polymerase α. Following reports from several laboratories that highly purified preparations of DNA polymerase α contained DNA primase activity, Wang et al. (1984) demonstrated a very tight association of these two enzymes with an immunoaffinity column of monoclonal antibodies against KB cell DNA polymerase α. DNA primase is much more easily denatured than DNA polymerase α, and in HeLa cells, 200-fold purification of DNA primase does not separate it from DNA polymerase α (Gronostajski et al., 1984).

The existence of multiple forms of DNA polymerase α is well documented [for example, Ottiger et al. (1984), Ono et al. (1978), Adams et al. (1973), Enomoto et al. (1983), and Yagura et al. (1983)]. Several studies have demonstrated that one of the DNA polymerase α forms is complexed with DNA primase (Conaway et al., 1982; Gronostajski et al., 1984; Nishizawa et al., 1983; Plevani et al., 1984; Shioda et al., 1982; Tseng et al., 1982; Wang et al., 1984; Yagura et al., 1983; Yoshida et al., 1983), and the consensus is that there is little free DNA primase activity in the cell, with most of it occurring as a tight complex with one of the forms of DNA polymerase α. Although the relationship to cell proliferation of DNA polymerase α is well-known, there have been only four studies of the relationship of DNA primase. DNA primase activity was found to correlate with DNA synthesis in spleen and cardiac muscle cells during postnatal development (Kozu et al., 1982). The activity of the DNA polymerase α–DNA primase complex increased during liver regeneration (Philippe et al., 1986; Tubo & Berezney, 1987). The amount of the DNA polymerase α–DNA primase complex purified from synchronized cells was 8-fold greater during the S phase of the cell cycle than during G_2 (Vishwanatha et al., 1986).

The nuclear matrix is the residual proteinaceous structure obtained after mild nuclease treatment of nuclei followed by washing with low-salt then high-salt buffer (Berezney, 1980). Even though such treatments remove more than 90% of bulk DNA, greater than 50% of nascent DNA remains tenaciously bound (Pardoll et al., 1980; Smith & Berezney, 1983; Valenzuela et al., 1983; Foster & Collins, 1985). The nascent DNA can be "chased" from the matrix into bulk DNA (Smith & Berezney, 1983; J. M. Collins, unpublished observations). HeLa matrices prepared by *Eco*RI digestion of nuclei have been shown to have a 40-fold enrichment of replicating forks

†Supported in part by USPHS Grant CA-24158 awarded by the National Cancer Institute.
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0006-2960/87/0426-5600$01.50/0

(Valenzuela et al., 1983). From observations such as these came the idea that the nuclear matrix is the site of DNA synthesis. Indeed, isolated nuclear matrices are capable of in vitro DNA synthesis (Smith & Berezney, 1983; Foster & Collins, 1985). This structure, whether called the nuclear matrix, cage, or scaffold, has also been implicated in transcription (Jackson et al., 1981) and RNA processing (Ciejek et al., 1982).

DNA is apparently arranged in the nucleus as domains or loops, ranging in size from 60 to 200 kilobases, attached to the nuclear matrix (Pardoll et al., 1980; Vogelstein et al., 1980; McCready et al., 1980). The estimated number of these attachment sites ranges from 60 000 to 120 000 depending on the cell type (Berezney & Bucholtz, 1981). The small amount of original DNA that remains bound after nuclease digestion and salt treatment presumably represents the attachment fragments of the loops (Berezney & Bucholtz, 1981). Over the S phase of HeLa, there are two bursts, or peaks, of DNA synthesis (Collins, 1978; Collins et al., 1980; Foster & Collins, 1985). This has led to the terms "early" and "late" DNA synthesis by Holmquist et al. (1982) and Goldman et al. (1984). It has been suggested that these early and late DNA syntheses represent two functionally distinct domains in the nucleus and that the dissociation of the replicative machinery from one domain to the other causes the two peaks (Holmquist et al., 1982). It is interesting to speculate that these domains may be functionally distinct parts of the nuclear matrix. In support of this idea is the observation that in vitro DNA synthesis by the nuclear matrix is also biphasic over S phase (Foster & Collins, 1985).

We have reported that from 10 to 30% of the total cellular DNA polymerase α remains bound to nuclear matrices prepared from low- then high-salt extraction of digested nuclei such that they retain 80–95% of nascent DNA but only 4–8% of bulk DNA (Foster & Collins, 1985). This polymerase activity was inhibited by *N*-ethylmaleimide, ddATP,[1] aphidicolin, and the monoclonal antibody designated SJK-20 and hence appears to be DNA polymerase alpha (Foster & Collins, 1985). The amount of DNA polymerase α bound to these DNA-poor matrices over the S phase of synchronized HeLa cells correlated with the biphasic rates of cellular DNA synthesis, whereas there was no such correlation with "total cellular" or with "nuclear" DNA polymerase α (Foster & Collins, 1985). We later observed that whereas most of DNA polymerase α activity can be recovered in soluble cytoplasmic plus nuclear extracts, the majority of DNA primase activity remained insoluble (Wood & Collins, 1986). Upward of 80–85% of the DNA primase activity was recovered as localized to DNA-poor, 2 M salt resistant nuclear matrices even after 13 cycles of low- then high-salt extraction (Wood & Collins, 1986).

As it has been proposed (Ottiger & Hubscher, 1984) that the different forms of DNA polymerase α may have different replicative functions, it seemed worthwhile to determine which form, or forms, is bound to the nuclear matrix. The ability to dissociate the DNA polymerase α and DNA primase activities from the matrix with octyl β-D-glucoside (Wood & Collins, 1986) permits this question to be answered. We now report that upward of 90% of the DNA polymerase α activity that remains bound to DNA-poor nuclear matrices is in the form of a DNA polymerase α–DNA primase complex. Furthermore, the amount of this complex bound to the nuclear matrix varies over the cell cycle.

Experimental Procedures

Materials. All tissue culture supplies were obtained from Flow Laboratories. HeLa cells were obtained from Dr. Thoru Pederson, Worchester Foundation for Experimental Biology, Shrewsbury, MA. [2,8-^{3}H]ATP (32 Ci/mmol) and [α-^{32}P]dATP (650 Ci/mmol) were obtained from ICN. [*methyl*-^{3}H]TTP (80 Ci/mmol) was obtained from New England Nuclear. ATP, deoxynucleoside triphosphates, dithiothreitol (DTT), β-mercaptoethanol (βME), dideoxyadenosine triphosphate (ddATP), octyl β-D glucoside, deoxycholate, calf thymus DNA, DNase I, and aphidicolin were obtained from Sigma. Triton X-100 was from Eastman. Poly(dT) was from Pharmacia. *Escherichia coli* DNA polymerase I (Klenow fragment) was obtained from United States Biochemicals. ATPγS was from Boehringer Mannheim. Activated calf thymus DNA was prepared as described previously (Foster & Collins, 1985).

Cell Culture. HeLa cells (0.5×10^6 cells/mL) were maintained in spinner culture at 37 °C. Cells were fed every 48 h with Joklik's modified Eagle's minimal essential medium containing 10% fetal calf serum and 1.25 μg/mL Fungizone and monitored for mycoplasma contamination as described (Collins, 1978).

Cell Synchronization. Cell synchrony was achieved by a modification of the method of Collins (1978). Cells were maintained on 2 mM thymidine for 14 h and then resuspended in fresh media and allowed to grow in the absence of thymidine for 9 h. Cells were exposed to a second 2 mM thymidine block for 14 h and then released from the block, centrifuged, and resuspended in fresh media. Under these conditions, greater than 90% of the cells were at the G_1/S boundary, as judged by the subsequent movement of more than 90% of the cells through the S phase (see Table II). The movement of the cells through S phase was monitored by flow cytometry of cellular DNA–propidium iodine fluorescence (Collins, 1978).

Preparation of Nuclei and "Cytoplasm". Nuclei and cytoplasm were isolated as described previously (Foster & Collins, 1985). HeLa cells [$(2–4) \times 10^6$/mL] were held for 2 h at 4 °C in 25 mL of a buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1 mM $MgCl_2$. The cells were disrupted by a Dounce homogenizer (~30 strokes) such that essentially no intact cells were noted by microscopic visualization. The nuclei were isolated as the pellet and the cytoplasm was isolated as the supernatant, following centrifugation of the homogenate at 10000 rpm in a Sorvall RC2-8, Type SS-34 rotor for 10 min. The nuclei were further washed 3 times with the above low-salt buffer and the washings combined with the cytoplasmic fraction. Bensch et al. (1982) have demonstrated that with the proper immunological techniques DNA polymerase α is localized exclusively in the nucleus. Hence, to avoid the use of the misleading term "cytoplasmic fraction", we refer hereafter to that fraction released during nuclei isolation combined with the "nuclear fraction" released during the initial low-salt washing of nuclei as the "soluble" fraction.

Preparation of Nuclear Matrix. Isolation of the nuclear matrix was accomplished by a modification of the method of Pardoll et al. (1980). In previous publications from this laboratory dealing with the nuclear matrix, the matrices were prepared by endogenous digestion, which necessitated a 16-h period due to the low nuclease activity in HeLa nuclei (Foster & Collins, 1985). In preliminary experiments we noticed that about 5 times more DNA polymerase α and about twice as much protein were retained when DNase digestion was the

[1] Abbreviations: DTT, dithiothreitol; βME, β-mercaptoethanol; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); PBS, phosphate-buffered saline; ddATP, dideoxyadenosine triphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

method of choice. Accordingly, in all of the experiments reported herein, DNase digestion was used to prepare nuclear matrices. Nuclei, isolated as described above, were digested with 50 μg/mL DNase I for 30 min at 37 °C, unless otherwise noted. Low-salt extraction of digested nuclei consisted of three washings with PBS and then three washings with TMP (10 mM Tris-HCl, pH 7.5, 0.2 M $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride), followed by resuspension in 10 mL of TMP. Next, the nuclei were extracted with high salt by the slow addition over 1 h of 4 mL of a 5 M NaCl–TMP solution to a final 2 M NaCl concentration. Following centrifugation of this homogenate (as described above for nuclei isolation), excess salt was removed from the pellet by washing 3 times with phosphate-buffered saline (PBS), 4 times with a different low-salt buffer (20 mM KPO_4, pH 7.5, 2 mM BME), then 3 times with a high-salt buffer (0.5 M KPO_4, 2 mM BME), and finally 3 times with the low-salt buffer. The final nuclear matrix preparations were resuspended in PBS. Electron microscopy revealed that most of the chromatin fibers present in isolated nuclei were absent from these nuclear matrix preparations. These preparations now had the appearance of a proteinaceous, scaffold-like structure similar to that seen by others (Berezney, 1980; data not shown).

Incorporation of [^3H]- and [^{14}C]Thymidine into Nascent and Bulk DNA of Nuclear Matrices. For nascent DNA, cells were exposed to 10 μCi/μL [^{3}H]thymidine for 5 min at 37 °C prior to preparation of nuclear matrices. For bulk DNA, cells were cultured with 1 μCi/mL [^{14}C]thymidine for 6 h, rinsed to remove radioactivity, and subsequently cultured on media without thymidine for 24 h prior to preparation of nuclear matrices.

Solubilization of Matrix-Bound DNA Primase and DNA Polymerase α. Following incubation in the low-salt buffer for 24 h at 0 °C, the extensively washed nuclear matrix preparation was sonicated 5 times for 15 s at 20-s intervals and allowed to remain on ice for 2 h. This homogenate was then incubated with 0.5 M KCl and 22 mM octyl β-D-glucoside for 1 h at 0 °C. Subsequent to centrifugation at 40000 rpm in a Beckman L3-50 centrifuge, Type 65 rotor, at 4 °C, the supernatant and pellet were collected. The pellet, representing residual matrix, was washed 3 times with PBS and then resuspended in 5 mL of PBS. The supernatant, containing solubilized matrix components, was then extensively dialyzed against PBS. This supernatant is hereafter referred to as the "dissociated" fraction, to avoid confusion with the soluble fraction.

Enzyme Assays. DNA polymerase α activity was measured by a method modified from Edenberg et al. (1978). Incorporation of [^{3}H]TMP into activated DNA was measured in a reaction mixture (250 μL) containing 10 mM Tris-HCl, pH 8.0, 10 mM MgOAc, 2 mM DTT, 0.67 mg/mL bovine serum albumin, 50 μM each of dATP, dGTP, dCTP, and [^{3}H]TTP (1.2 Ci/mmol), 500 μg/mL activated DNA, 100 μM ddATP, and 50 μL of the sample to be assayed. Inclusion of ddATP under these conditions provides a greater than 85% inhibition of polymerase β. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 5 mL of cold 1 N HCl–1% $NaPP_i$. The reaction mixture was collected on Whatman GF/C filters and washed successively with 3 mL of cold 1 N HCl–1% $NaPP_i$ and 3 mL of cold 90% ethanol, and the radioactivity was determined in a scintillation counter. Under the conditions described, incorporation of labeled TMP is linear over 60 min. One unit of DNA polymerase α activity is defined as that amount which catalyzes the incorporation of 1 nmol of TMP into activated DNA in 1 h at 37 °C. DNA primase was assayed in a coupled reaction involving the incorporation of labeled dAMP into poly(dT), a single-stranded template, by DNA polymerase α and the Klenow fragment of *E. coli* polymerase I (hereafter referred to as the Klenow fragment) following the formation of the requisite ribonucleotide primer by DNA primase. The reaction mixture (250 μL) consists of the following components: 25 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 4 mM DTT, 50 μg/mL poly(dT), 50 μM ATP, 25 μM [α-^{32}P]dATP (3 Ci/mmol), Klenow fragment (1 unit/mL), 200 μg/mL bovine serum albumin, and 50 μL of the material to be assayed. The reaction is terminated, and the products are processed as described for DNA polymerase α above. One unit of DNA primase activity is defined as that amount which catalyzes the incorporation of 1 nmol of dAMP into poly(dT) in 1 h at 37 °C.

Protein Determination. Protein concentrations were determined by the method of Lowry et al. (1951).

Heat Denaturation. Matrix-bound, dissociated, and soluble enzyme preparations were heated in 10 mM Tris-HCl, pH 8.0, and 10 mM NaOAc at 40 °C for various times, quickly chilled on ice, and assayed for enzyme activity as described above.

Sucrose Gradient Centrifugation. Soluble and dissociated preparations were subjected to sedimentation in 12.5-mL sucrose gradients, as previously described (Collins, 1974b). The *s* values were determined with rabbit muscle aldolase (7.35 S) as a marker. The resulting data were analyzed by a general nonlinear least-squares computer program to quantitate the amounts of activity in various Gaussian distributions. The details have been described elsewhere (Collins, 1978).

RESULTS

Preliminary Experiments. It has been reported that exposure of isolated nuclei to temperatures above 35 °C causes the artifactual insolubilization of p62c-myc, v-myc, and v-myb proteins such that they appear to be isolated as "bound" to the number matrix (Evan & Hancock, 1985). To ascertain whether DNA polymerase α and DNA primase exhibited similar behavior, the effect of temperature of DNase digestion on nuclei from which nuclear matrices were subsequently prepared was determined. As can be seen in Table I, after 240 min at 4 °C, only 6% of bulk DNA is retained while 80% of nascent DNA remains bound, and after 30 min at 37 °C, only 5% of bulk DNA is retained while 94% of nascent DNA remains bound. This enrichment of nascent DNA is characteristic of DNA-depleted nuclear matrices [i.e., Smith and Berezney (1982)] and is apparently not affected by temperature (Table I). Under these conditions, the amount of DNA polymerase α and DNA primase activity that is retained appears to be virtually unaffected by temperature (Table I). The experiment was duplicated with an error range of about 5%. In other experiments, undigested nuclei were extracted with first low salt and then high salt to prepare DNA-rich nuclear matrices, which were subsequently digested at 0 and 37 °C. The results were remarkably similar to those of Table I, again strongly suggesting that elevated temperatures do not cause artifactual binding of DNA polymerase α and DNA primase to the nuclear matrix (data not shown).

Razin et al. (1985) reported that a low-salt extraction step abolished the "retention" of transcriptionally active DNA by the nuclear matrix, suggesting that an artifactual "binding", perhaps due to an increase in insolubilization, may occur when a single high-salt step is used. Although the nuclear matrices used for the data in Table I were extracted with low- and then high-salt buffer, we also examined the effects of omission of the low-salt extraction step on the retention of DNA polym-

Table I: Effect of Temperature of Digestion on Retention of Polymerase α and Primase Activities by the Nuclear Matrix[a]

	percent of total at 4 °C				percent of total at 37 °C			
time	bulk DNA[b]	nascent DNA[c]	Pol α	primase	bulk DNA[b]	nascent DNA[c]	Pol α	primase
0	100[d]	100[e]	100[f]	100[g]	100[d]	100[e]	100[f]	100[g]
5	88	98	99	98	33	99	96	96
15	65	97	98	98	8	96	92	93
30	29	92	98	95	5	94	91	91
60	19	89	97	93				
120	6	86	98	93				
240	6	81	98	90				

[a] Routinely isolated nuclear matrices were prepared from nuclei digested at 4 or 37 °C as described under Experimental Procedures. [b] Bulk DNA = 6-h pulse/24-h chase with [^{14}C]thymidine; see Experimental Procedures. [c] Nascent DNA = 5-min pulse with [^{3}H]thymidine; see Experimental Procedures. [d] 100% = 6.4 × 10^5 cpm per 10^6 nuclei for ^{14}C-labeled bulk DNA. [e] 100% = 2.6 × 10^4 cpm per 10^6 nuclei for ^{3}H-labeled nascent DNA. [f] 100% = 85 units of DNA polymerase α activity. [g] 100% = 7 units of DNA primase activity.

erase α and DNA primase. When the low-salt step was omitted, matrices prepared by 240 min of digestion at 4 °C contained 84 units of DNA polymerase activity and 7.4 units of DNA primase activity compared to 81 and 7.1 units, respectively, for matrices first treated with low salt, differences that are not significant.

In another series of preliminary experiments, DNA-poor nuclear matrices ("control matrices") containing 81 units of DNA polymerase α and 7.1 units of DNA primase were mixed with soluble extract containing 552 units of DNA polymerase α and 9.2 units of DNA primase and then once again subjected to salt treatment. After a low- and then high-salt extraction, the matrices contained 74 units of DNA polymerase α and 6.8 units of DNA primase, and after a single high-salt extraction, they contained 76 units of DNA polymerase α and 7.5 units of DNA primase. In a different experiment the same amount of the same soluble extract was added to an equivalent amount of nuclei before DNase digestion at 4 °C followed by low- and then high-salt extraction. The resulting nuclear matrices contained 87 units of DNA polymerase α and 7.6 units of DNA primase. When the low-salt extraction step was omitted, the matrices resulting from this mixing experiment contained 92 units of DNA polymerase α and 8.0 units of DNA primase, an increase of 10% over the "control matrices". These preliminary data strongly suggest that the DNA polymerase α and DNA primase bound to the nuclear matrix are not an artifact of temperature or salt under our experimental conditions (DNase digestion at 37 °C, 19 cycles of low- and high-salt extraction—see Experimental Procedures).

DNA Polymerase α and DNA Primase Activities Dissociated from the Nuclear Matrix. The sedimentation pattern of DNA polymerase α and DNA primase activities dissociated from the nuclear matrix and in the soluble extracts of log-phase cells are presented in Figure 1. The majority of the DNA polymerase α and DNA primase activities dissociated from the matrix cosediments as a band of about 10.5 S (Figure 1A), which is taken to be the DNA polymerase α–DNA primase complex. This complex constitutes 85% of the total DNA polymerase α and 95% of the total DNA primase activities bound to the matrix. Approximately 10% of the DNA polymerase α sediments free of DNA primase, at a value of 7.5 S. There is a minor band comprising DNA polymerase α–DNA primase activities, constituting 5% of the total of each activity bound, which sediments with a value of 13 S. That identical *s* values were obtained for the corresponding enzymatic activities of the soluble extract (Figure 1B) suggests that the values for the dissociated activities (Figure 1A) are not artifactual results of high-salt or octyl β-D-glucoside treatment. When the dissociated activities were chromatographed with anionic fast-pressure liquid chromatography (Pharmacia Mono-2 column) or with DEAE-cellulose, the proportions of

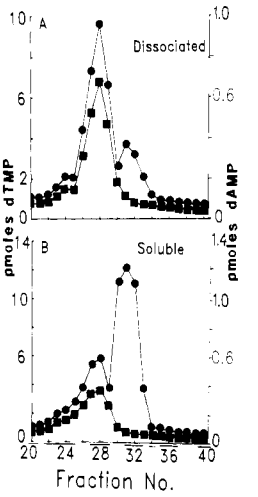

FIGURE 1: Sucrose density gradient sedimentation of polymerase α and primase activities dissociated from the nuclear matrix. Approximately 0.5 mL of enzyme activities constituting 15 μg of protein was layered onto 12.5 mL of 5–20% neutral sucrose gradients. Rabbit muscle aldolase, 7.35 S, was used as a marker. (A) Enzyme activities dissociated from the nuclear matrix; (B) enzyme activities in the soluble fraction. (●) DNA polymerase α, picomoles of dTMP incorporated; (■) DNA primase, picomoles of dAMP incorporated.

the DNA polymerase α–DNA primase complex and the DNA primase free DNA polymerase α were similar (data not shown) to those in the sucrose gradients of Figure 1. It should be stressed that the log-phase cells used for this series of experiments constituted a mixture of 58% G_1, 24% S-phase, and 18% G_2M cells; hence, there is no reason to expect that similar proportions of bound activities would occur over the cell cycle of synchronized cells.

Effect of ATP, KCl, Heat, Mg^{2+}, and pH on DNA Primase. DNA primase was examined as nuclear matrix bound, dissociated from the matrix, and soluble activities. The matrix-bound activity differs in ATP dependence, with an optimum at 0.05 mM, whereas both the soluble and dissociated activities have optima at 0.1 mM (Figure 2A). The three activities also exhibited differential inhibition by KCl, with 50% inhibition occurring at 100 mM for the matrix-bound activity and at 65 mM for the soluble and dissociated activities (Figure 2B). At 200 mM, the inhibition was about 80%, 90%, and 90% for the matrix-bound, soluble, and dissociated activities, respectively (Figure 2B). The matrix-bound activity had greater heat stability that the dissociated and soluble activities (Figure 2C). After 5 min at 40 °C, the matrix-bound DNA primase had about 50% remaining activity as compared to 20% and 20% for the dissociated and soluble activities, respectively (Figure 2C). These experiments were repeated 3 times with an error range of about 6%, a range encompassed within the symbols of Figure 2. The three activities were

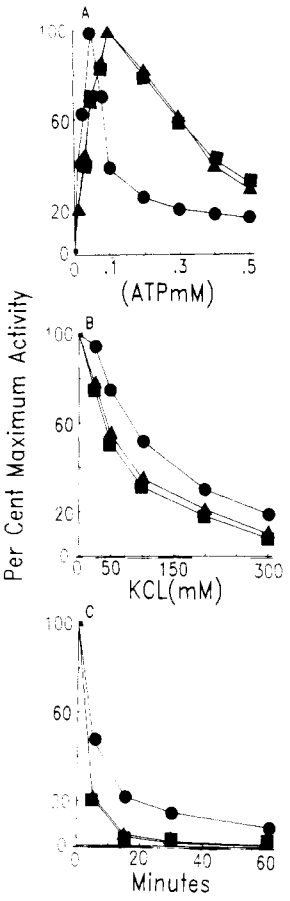

FIGURE 2: Effects of ATP, KCl, and heat on DNA primase. Primase was assayed as described under Experimental Procedures. (A) ATP dependence; (B) KCl inhibition; (C) heated at 40 °C. (●) Activity bound to the nuclear matrix; (■) activity dissociated from the nuclear matrix; (▲) activity in soluble fraction.

similar in Mg^{2+} dependence, with about 35% stimulation occurring at 0.5 mM, a level of activity that was maintained up to 3 mM (data not shown), and in pH optima, with a broad pH dependence centered at about pH 8.0 (data not shown). When DNA primase activity was measured directly by the incorporation of [^{3}H]AMP into matrix DNA (matrix fraction) or poly(dT) (dissociated and soluble fractions), the results were similar to those seen in Figure 2 (data not shown).

Under conditions of heat denaturation similar to those of Figure 2C, matrix-bound, dissociated, and soluble DNA polymerase α activities were much less heat sensitive than DNA primase, with the matrix-bound activity only slightly more stable than the other two (data not shown). Activities of 99%, 80%, 47%, and 28% remained after heating for 5, 15, 30, and 60 min, respectively (data not shown). Furthermore, when the dissociated activity was heated for 30 min, conditions under which only 2% of the DNA primase activity remains (Figure 2C), subsequent sedimentation in sucrose gradients yielded similar proportions of 13S, 10.5S, and 7.5S forms as seen in Figure 1, indicating that no conversion from one form to another had occurred (data not shown).

Synchronized Cells. In order to determine whether the proportions of the activity of the DNA polymerase α–DNA primase complex relative to the DNA primase free DNA polymerase α change over the cell cycle, HeLa cells were synchronized at the G_1/S boundary and then allowed to progress through the cycle. As can be seen, the cells proceeded through the cycle as a highly synchronous population (Table II). The cells began to enter S at 2 h and progressed through S until 8–9 h whereupon they entered G_2M. At 10 h they began to divide and enter G_1, through which they traveled until 22 h whereupon they began to reenter the S phase.

Table II: Progression of Synchronized Cells through the Cell Cycle[a]

hours after release	cell cycle phase		
	G_1	S[b]	G_2M
0	99 (1)[c]	1	
1	99[c]	1	
2	5	95 (1.1)	
3	2	98 (1.2)	
4	2	98 (1.3)	
5	3	97 (1.5)	
6	2	98 (1.6)	
7	1	99 (1.7)	
8	2	98 (1.9)	
9		20 (1.9)	80 (2)
10	10		90
16	99		1
18	99		1
20	99		1
22	95	5 (1.1)	
24	5	95 (1.1)	

[a] At the times indicated, portions of cells released from double-thymidine blockade were analyzed for DNA content by flow cytometry as described under Experimental Procedures. [b] The relative DNA content of G_1 cells is always 1, and that of a G_2M cell is always 2.0. The DNA contents of the S-phase cells, relative to a G_1 cell, 16.1 pg, are given in parentheses; these values range from 1.01 to 1.99. [c] Actually G_1/S boundary.

Dissociated DNA Polymerase α and DNA Primase over the Cell Cycle. DNA polymerase α and DNA primase were measured over the entire cell cycle as activities dissociated from the nuclear matrix and sedimented through sucrose gradients to resolve the multiple DNA polymerase α forms. As the amounts of the dissociated activities subsequently sedimented in sucrose gradients varied considerably over the cycle, the data are presented as fraction of maximum activity in each gradient, with maximal DNA polymerase α set to 1.0 and maximal DNA primase set to 0.7, except for panels 9H and 18H where DNA primase was very low (Figure 3). DNA primase activity was present from 0 to 8 h (when cells were going through S phase), was very low from 9 to 20 h (when cells were going through G_2M and G_1), and was present again at 22–24 h (when cells were reentering S phase) (Figure 3). Although the *total* amounts of activity (given in the legend of Figure 3) varied, the *ratio* of DNA primase to DNA polymerase α activity was almost constant, varying from 0.64 to 0.71, from 0 to 16 h. It is not readily apparent in this figure, but during late G_1, 16–20 h, DNA polymerase α, but not DNA primase, increased and then decreased in activity (see legend to Figure 3 and Figure 4A).

DNA Polymerase α and DNA Primase over the Cell Cycle. The variation in the amounts of DNA polymerase α and DNA primase activities dissociated from the nuclear matrix over the cycle, given in the legend of Figure 3, is presented in Figure 4A. The biphasic nature of these activities over the S phase, from 0 to 8 h (Figure 4A), is strikingly similar to the biphasic nature of the rates of DNA synthesis over the same period (Collins, 1978; Collins et al., 1980; Foster & Collins, 1985). Both enzyme activities decline to a relatively low level during G_2M, from 9 to 10 h. Surprisingly, DNA polymerase α, but not DNA primase, shows a "burst" of activity during late G_1, 15–18 h, which may be related to postreplicative repair (Djordjevic et al., 1969). As the cells begin to reenter the S phase, from 22 to 24 h, each enzyme again increases in activity (Figure 4A). The amounts of matrix-bound (undissociated) activities over the cycle (Figure 4B) had a pattern similar to those of the dissociated activities in Figure 4A. The activities over the cycle of DNA polymerase α and DNA primase in

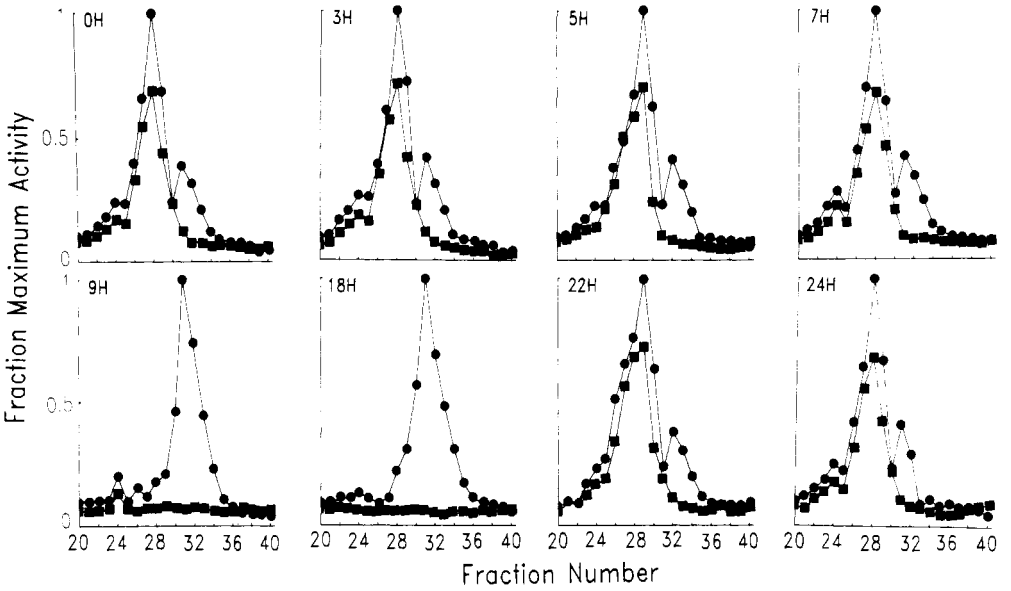

FIGURE 3: Multiple forms of DNA polymerase α over the cell cycle. Synchronized cells were harvested at various times during the cell cycle for preparation of nuclear matrices from which enzyme activities were subsequently dissociated and 15 μg of protein was sedimented through sucrose gradients as described in the legend of Figure 1. The time after release is given in the upper left of each panel. (●) DNA polymerase α activity; (■) DNA primase activity. As the amounts of activity varied considerably, the data are graphed as fraction of the maximal value of each gradient, with the maximum DNA polymerase α fraction set to 1.0 and the maximum DNA primase fraction set to 0.7 to prevent overlap of the two activities, except in panels 9H and 18H where DNA primase was very low; in panels 9H and 18H, the DNA primase values were set to fraction above background. The amounts of activity in picomoles recovered from all of the gradients, including those not shown, were, at the times indicated, for DNA polymerase α, and DNA primase, respectively, as follows: (0 h) 30.7 and 2.16; (2 h) 102.5 and 7.28; (3 h) 160.9 and 11.21; (4 h) 143.5 and 10.12; (5 h) 84.5 and 5.91; (6 h) 117.8 and 7.89; (7 h) 256.2 and 17.50; (8 h) 53.8 and 3.56; (9 h) 5.1 and 0.25; (12 h) 3.1 and 0.21; (14 h) 10.2 and 0.20; (16 h) 43.6 and 0.21; (18 h) 74.3 and 0.21; (20 h) 10.2 and 0.21; (22 h) 4.4 and 0.20; (23 h) 128.1 and 8.71; (24 h) 222.9 and 15.38.

the soluble fraction (Figure 4C) showed little correlation with early and late DNA synthesis and never decreased to relatively low values as did the matrix-bound activities. In separate experiments, we observed no increases in DNA polymerase β, the so-called repair polymerase, during these time periods (data not shown). The experiments depicted in Figure 4 were repeated several times with essentially the same results.

Discussion

DNA polymerase α has been detected on the nuclear matrix of rat liver (Smith & Berezney, 1983; Nishizawa et al., 1984), SV-40-infected african monkey cells (Jones & Su, 1982), Ehrlich Ascites cells (Chanpu et al., 1984), chick embryo (Yamamoto et al., 1984), and HeLa cells (Foster & Collins, 1985). The amounts usually range from 5 to 30% of the nuclear total, depending on the preparative methods used. Smith and Berezney (1983) found much more DNA polymerase α on the matrix in regenerating liver than in control liver, suggesting a replicative function for the translocative binding of DNA polymerase α to that structure.

It would seem from our preliminary experiments that the retention of DNA polymerase α and DNA primase activities by DNA-poor nuclear matrices is not an artifact resulting from the use of temperatures above 35 °C during DNase digestion (Evan & Hancock, 1985), as similar amounts of enzyme activities resulted whether digestion was performed at 4 or 35 °C (Table I). In addition, the temperature had little effect on the retention of nascent DNA after removal of most of the bulk DNA (Table I). In a similar manner, the omission of a low-salt extraction step prior to high-salt extraction had little effect on the retention of enzyme activities, resulting in only a 4% increase (see Preliminary Experiments). Indeed, in experiments where nuclei were mixed with soluble enzymatic extracts prior to preparation of nuclear matrices, the resulting matrix-bound activities were almost the same as if no soluble activities had been added (see Preliminary Experiments).

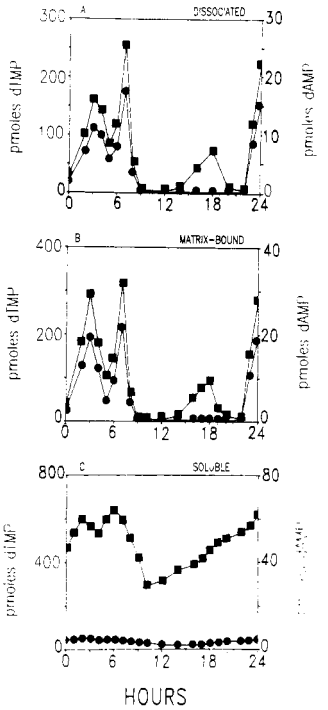

FIGURE 4: Enzymatic activities of DNA polymerase α and DNA primase over the cell cycle. Synchronized cells were harvested at various times for preparation of nuclear matrices and subsequent determination of DNA polymerase α and DNA primase activities. (A) Summation of the activities in dissociated preparations sedimented in sucrose gradients, from the legend of Figure 4; (B) activities bound to the nuclear matrix; (C) activities in the soluble fraction (cytoplasmic + nuclear washes). (■) DNA polymerase α, picomoles of dTMP incorporated; (●) DNA primase, picomoles of dAMP incorporated.

DNA primase bound to the nuclear matrix has a lower ATP dependence (Figure 2A), is less inhibited by KCl (Figure 2B), and is less heat sensitive (Figure 2C). The possibility that the

bound enzyme might be a novel form of DNA primase seems remote since the activity dissociated from the matrix is similar to that of the soluble activity in s value (10.5 S, Figure 1), in ATP dependence (Figure 2A), in KCl inhibition (Figure 2B), and in heat stability (Figure 2C). It seems more likely that the differences result from an altered conformation when DNA primase is bound, although the only way to prove this would be to bind dissociated DNA primase back onto the matrix with resultant matrix-bound characteristics. Thus far, we have been unsuccessful in doing this.

The ratio of DNA polymerase α to DNA primase activities bound to the nuclear matrix is remarkably constant over the cell cycle, except during mid to late G_1 (16–18 h) when there is a small but significant increase in DNA polymerase α activity without a corresponding increase in DNA primase activity (Figure 4A,B). In this regard, it has been reported by Djordjevic et al. (1969) that there is a burst of postreplicative repair (also called "spontaneous unscheduled DNA synthesis") during late G_1. If the increase in matrix-bound DNA polymerase α activity (Figure 4A,B) has a repair function, then it would follow that matrix-bound DNA primase is not necessary.

Our present results with DNase I digested nuclei agree with those of Vishwanatha et al. (1986), in that 70–80% of the total DNA polymerase α activity is extracted from undigested nuclei by low-salt extractions, and with our previous report (Wood & Collins, 1986) that only 20–40% of the total DNA primase is extracted, with the remaining 60–80% recovered in the salt-resistant nuclear matrix fraction. Our observations on the levels of DNA polymerase α–DNA primase activity in the soluble fraction at the G_1/S boundary, during two parts of S phase, and during G_2 also agree with those of Vishwanatha et al. (1986) and extend those data to include the entire cell cycle (Figure 4C) and the levels bound to the nuclear matrix (Figure 4A,B). Vishwanatha et al. (1986) reported molecular masses for their 6S and 10.3S DNA polymerase α forms of 220 kDa and 640 kDa, respectively. Although three forms of DNA polymerase α can be detected in HeLa cells, in general the two major forms, 7.5S and 10.5S, constitute about 90% of the total α activity (Adams et al., 1973; Krauss & Lin, 1982; Enomoto et al., 1983; Gronostajski et al., 1984). The α portion of the two major DNA polymerase α forms in HeLa cells may be different polypeptide species, rather than a single species which occurs complexed with DNA primase or not, as Yagura et al. (1986) have presented convincing data that in mice the 10.5S DNA primase associated form resolves into a 115-kDa catalytic polypeptide distinct from the 72-kDa catalytic polypeptide of the 7.3S DNA primase free form. Different replicative functions have been proposed for the DNA primase free form of DNA polymerase α and the DNA polymerase α–DNA primase complex in HeLa cells (Ottiger & Hubscher, 1984). These authors suggested that the free form functions in replication of the leading (forward) strand whereas the DNA polymerase α–DNA primase complex functions in replication of the lagging (retrograde) strand. If so, one might predict that greater amounts of the DNA polymerase α–DNA primase complex would be needed due to the greater number of chain initiations required on the lagging strand. Seemingly at variance with this is our estimation that the DNA polymerase α–DNA primase complex constitutes only about 30% of the total DNA polymerase α in the cell. However, in the nuclear matrix fraction, the DNA polymerase α–DNA primase complex does predominate, constituting 90% of the total DNA polymerase α in that fraction (Figure 1A). Since replication is proposed to occur at the level of the nuclear matrix (Smith & Berezney, 1982; Pardoll et al., 1980; Valenzuela et al., 1983; Foster & Collins, 1985), the preponderance of the DNA polymerase α–DNA primase complex at that level, as a reflection of greater demands for chain initiation, might be expected. In this regard, while this paper was under review, Tubo and Berezney (1987) reported that rat liver nuclear matrix retains ribonuclease H activity, 3′ to 5′ exonuclease activity, and DNA methylase activity in addition to DNA polymerase α and DNA primase. They observed an increase in the amount of DNA polymerase α, ribonuclease H, DNA methylase, and DNA primase bound to the nuclear matrix before entry into the DNA synthetic phase, and thus, our data confirm theirs, with respect to DNA primase, in a different system. However, they observed that DNA primase activity increases 6-h posthepatectomy, well before the onset of S phase, while we note an increase just before S phase. Also, they found that only about 20% of the nuclear DNA primase is retained after salt extraction, in contrast to the 60–80% observed by us. As the salt-extraction conditions were very similar, we can offer no explanation for these differences between rat liver and HeLa cells.

The biphasic nature of DNA replication in HeLa cells (Collins, 1978; Collins et al., 1980; Foster & Collins, 1985) has led to the terms early and late DNA (Holmquist et al., 1982; Goldman et al., 1984). Early and late DNA syntheses took on greater significance when Goldman et al. (1984) presented hybridization data with several DNA probes that suggested that most expressed housekeeping genes and most expressed tissue-specific genes are replicated as early DNA and most inexpressed genes are duplicated as late DNA. The dynamic domain hypothesis of Smith and Berezney (1982) predicted that a key factor in the onset of S phase is the translocation of DNA polymerase α from one domain in the nucleus (soluble) to another (matrix bound). The present work confirms that hypothesis and suggests that it is the translocation of the DNA polymerase α–DNA primase complex, rather than DNA primase free DNA polymerase α, that is that key factor. Furthermore, the biphasic nature of the activities of the complex over the S phase (Figures 3 and 4) is strikingly reminiscent of the biphasic nature of DNA replication in HeLa cells (Foster & Collins, 1985; Collins et al., 1980). We propose that it is the biphasic nature of the dynamic binding of the DNA polymerase α–DNA primase complex to the nuclear matrix that determines the biphasic nature of replication in HeLa cells. A supportive test of this proposal would be to observe a similar correlation between amounts bound to the matrix and rates of DNA synthesis in a different cell line. In HL60 cells synchronized by centrifugal elutriation, the replicative rates are not biphasic but almost constant over the S phase (Collins & Foster, 1983). We have observed that the amounts of enzymic activity of the DNA polymerase α–DNA primase complex bound to the nuclear matrix of HL60 cells are also almost constant over their S phase (data not shown). This will be the subject of a separate paper. Finally, the increase in the amount of activity of the matrix-bound DNA polymerase α–DNA primase complex as the cells reenter the S phase (Figure 4A,B) suggests that the binding of this complex to the nuclear matrix may be one of the determinants of the onset of S phase. This increase in matrix-bound activity could be in response to some presently unknown signal.

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