Enhanced Processivity of Nuclear Matrix Bound DNA Polymerase α from Regenerating Rat Liver†

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ABSTRACT: Translocation of DNA during in vitro DNA synthesis on nuclear matrix bound replicational assemblies from regenerating rat liver was determined by measuring the processivity (average number of nucleotides added following one productive binding event of the polymerase to the DNA template) of nuclear matrix bound DNA polymerase α with poly(dT)·oligo$(A)_{10}$ as template primer. The matrix-bound polymerase had an average processivity (28.4 nucleotides) that was severalfold higher than the bulk nuclear DNA polymerase α activity extracted during nuclear matrix preparation (8.9 nucleotides). ATP at 1 mM markedly enhanced the activity and processivity of the matrix-bound polymerase but not the corresponding salt-soluble enzyme. The majority of the ATP-dependent activity and processivity enhancement was completed by 100 μM ATP and included products ranging up to full template length (1000–1200 nucleotides). Average processivity of the net ATP-stimulated polymerase activity exceeded 80 nucleotides with virtually all the DNA products >50 nucleotides. Release of nuclear matrix bound DNA polymerase α by sonication resulted in a loss of ATP stimulation of activity and a corresponding decrease in processivity to a level similar to that of the salt-soluble polymerase (6.8 nucleotides). All nucleoside di- and triphosphates were as effective as ATP. Stimulation of both activity and processivity by the nonhydrolyzable ATP analogues adenosine 5′-*O*-(3-thiotriphosphate), 5′-adenylyl imidodiphosphate, and adenosine 5′-*O*-(1-thiotriphosphate) further suggested that the hydrolysis of ATP is not required for enhancement to occur. A degree of specificity of nucleotide activation was indicated by the inability of nucleoside monophosphates, the ATP analogues P^1,P^4-bis(5′-adenosyl) tetraphosphate and 5′-adenylyl methylenediphosphate, the ADP-nonhydrolyzable analogue adenosine 5′-*O*-(2-thiodiphosphate), and pyrophosphate to enhance either activity or processivity.

Several critical biological processes occurring within the eucaryotic cell nucleus have been shown to be closely associated with the nuclear matrix (Berezney, 1984). This dynamic proteinaceous nuclear framework is obtained following nuclease, salt, and detergent treatments of isolated nuclei (Berezney, 1984). Chromatin within the cell nucleus is organized into a series of supercoiled loops (Cook & Brezell, 1975; Benyajati & Worcel, 1976; Georgiev et al., 1978; Igo-Kemenes & Zachau, 1978), which are attached to the nuclear matrix of interphase nuclei (Wanka et al., 1977; Vogelstein et al., 1980; Berezney & Buchholtz, 1981a). A number of in vivo incorporation studies suggest that the sites of eucaryotic chromosomal replication are closely associated with the isolated nuclear matrix structure (Berezney & Coffey, 1975, 1976; Dijkwel et al., 1979, 1986; McCready et al., 1980; Pardoll et al., 1980; Berezney & Buchholtz, 1981b; Aelen et al., 1983; van der Valden et al., 1984; Carri et al., 1986; Razin et al., 1986).

It is proposed that during eucaryotic DNA replication DNA loops and nuclear matrix bound replicational complexes or replisomes translocate relative to each other (Dijkwel et al., 1979; McCready et al., 1980; Pardoll et al., 1980; Berezney & Buchholtz, 1981b). Consistent with this nuclear matrix replisome model, DNA polymerase α, DNA primase, and other replicative enzymes are associated with the nuclear matrix in a cell cycle dependent fashion (Smith & Berezney, 1980, 1982, 1983; Foster & Collins, 1985; Wood & Collins, 1986; Tubo & Berezney, 1987a), and at least a major portion of the in vitro DNA synthesis on endogenous matrix-attached DNA represents a continuation at in vivo initiated replicational forks (Tubo et al., 1985). Moreover, the nuclear matrix bound DNA polymerase α cosediments on sucrose gradients with several other replicative enzymes following solubilization from the matrix structure (Tubo & Berezney, 1987a). While the relationship of these matrix-solubilized multienzyme replicational complexes to functional sites of DNA replication in the cell nucleus remains to be clarified, it is interesting to note that recent immunocytochemical findings indicate that both DNA polymerase α and the sites of active in vivo replication are arranged in discrete granular clusters within the cell nucleus (Yamamoto et al., 1984; Nakamura et al., 1984, 1986). Moreover, the granular clusters or putative "replication centers" observed in intact cells were maintained following extractions used for obtaining nuclear matrix structures (Yamamoto et al., 1984).

In this study we have evaluated the ability of the nuclear matrix bound replisomes to translocate DNA by measuring the processivity of DNA polymerase α. Processivity is defined as the number of nucleotides added to a DNA template during a single binding–translocation–dissociation cycle of a polymerase (Bambara et al., 1978; Fay et al., 1981; Hockensmith & Bambara, 1981). We demonstrate that the matrix-bound enzyme is severalfold more processive in DNA synthesis then the corresponding salt-soluble polymerase of the cell nucleus. In addition, ATP and other nucleoside di- and triphosphates stimulate enzyme activity 2–3-fold and markedly enhance the processivity to DNA products up to full template length (1000–1200 nucleotides). Release of the polymerase from the nuclear matrix structure results in a loss in ATP stimulation of activity and a large decrease in processivity to a level similar to the bulk salt-soluble polymerase activity and most purified preparations of DNA polymerase α (Das & Fujimura, 1979; Fisher et al., 1979; Detera et al., 1981; Hockensmith &

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Bambara, 1981; Villani et al., 1981; Wierowski et al., 1983).

Experimental Procedures

Preparation of Nuclei and Nuclear Matrix. Nuclei and 2 M NaCl resistant nuclear matrix structures were prepared from two-thirds partially hepatectomized rats (Sprague-Dawley, 225–275 g, King Animal Labs) during the maximal period of in vivo DNA replication (22-h posthepatectomy), as described previously (Berezney & Coffey, 1977; Smith & Berezney, 1982) with slight modifications. In brief, nuclei following dense sucrose (2.2 M sucrose, 5 mM $MgCl_2$, 5 mM Tris,[1] pH 7.4, 1 mM PMSF) purification were resuspended to 2.0 mg of DNA/mL in 0.25 M sucrose, 5 mM $MgCl_2$, 5 mM Tris, pH 7.4 and 1 mM PMSF. Endogenously digested nuclei (37 °C for 45 min) were extracted 3 times with high-salt buffer (2 M NaCl, 10 mM Tris, pH 7.4, 0.2 mM $MgCl_2$, 1 mM PMSF) and twice with low-salt buffer (10 mM Tris, pH 7.4, 0.2 mM $MgCl_2$, 1 mM PMSF), yielding nuclear matrix. Washes with 0.4% (w/v) Triton X-100 in low-salt buffer were eliminated since some proteins associated with purified DNA polymerase α are dissociated by addition of nonionic detergent (Prichard & DePamphilis, 1983). Later studies revealed that the addition of Triton X-100 did not affect the activity or processivity properties reported in these studies for the nuclear matrix bound DNA polymerase α. Final nuclear matrix was stored at −20 °C in low-salt buffer made 50% in glycerol at one-third the original nuclear volume and generally contained 2–5% of the total nuclear DNA and 10–15% of total nuclear protein.

Solubilization of Nuclear Matrix Bound DNA Polymerase α. Nuclear matrices were isolated from 22-h regenerating liver as reported above except that rather than washing the final matrix pellets with low-salt buffer, they were suspended in the high-salt buffer to one-third the original nuclear volume and sonicated with two 20-s pulses at the lowest setting possible (Branson sonifier, Model 145W). The sonicate was centrifuged (11 000 rpm for 10 min) in a Eppendorf microfuge. The high salt sonicated supernatant (matrix extract) was carefully removed, made 50% in glycerol, and stored at −20 °C. The high salt sonicated pellet (matrix pellet) was washed twice with low-salt buffer (3000 rpm for 15 min), resuspended in low-salt buffer to the original aliquot volume, made 50% in glycerol, and stored at −20 °C.

Measurement of Activities of DNA Polymerase α on Exogenous Templates. DNA polymerase α reactions in a total volume of 50 μL contained 50 mM Tris, pH 7.2, 600 μg/mL DNase-free BSA, 15% glycerol, 2 mM DTT, 2 mM EGTA, 8 mM $MgCl_2$, 15 mM KCl, 0.1 mM PMSF, 1 mM ATP, and 5–15 μL of sample. In reactions using native DNA (activated calf thymus DNA), 80 μM dATP, dCTP, and dGTP and 2.4 μM [^{3}H]dTTP (9–60 Ci/mmol, ICN) were incubated at 37 °C for 30 min and terminated by precipitation with ice-cold 5% trichloroacetic acid (TCA). Acid-insoluble radioactivity was collected on 0.30 glass fiber filters (Schleicher & Schuell) and counted in liquiscent (National Diagnostics) with a Delta 300 liquid scintillation system (Tracor Analytic). Specifically primed templates were prepared by annealing homopolymer DNA template with the corresponding oligoribo– or oligodeoxyribonucleotide at 37 °C for 30 min. The homopolymeric templates poly(dT), poly(dA), and poly(dC) and the oligonucleotide primers were obtained from P-L Biochemicals while poly(A) was purchased from Miles Laboratories. The ATP analogues AMPPNP, AMPPCP, ATPγS, ADPβS, and AP_4A were purchased from Sigma, while ATPαS was from Boehringer Mannheim. All nucleotide solutions were neutralized (pH 7.0) with KOH before addition to the reaction mix.

Measurement of Processivity of DNA Polymerase α on Poly(dT)·Oligo(A)$_{10}$. Reactions to measure processivity (100 μL) contained 50 mM Tris, pH 7.2, 600 μg/mL DNase-free BSA, 15% glycerol, 2 mM DTT, 2 mM EGTA, 8 mM $MgCl_2$, 15 mM KCl, 0.1 mM PMSF, ribo- or deoxyribonucleotide at concentrations indicated in the figure legends, and 10–20 μL of sample. All nucleotide solutions were neutralized (pH 7.0) with KOH before addition to the reaction mix. The final concentration of radiolabeled dATP was 10 μM [^{3}H]dATP (27 Ci/mmol, ICN) or 10 μM [^{32}P]dATP (20 Ci/mmol, ICN). The poly(dT)·oligo(A)$_{10}$ template primer (1:1 template:primer ratio unless otherwise indicated) was 100 μM. The reaction was incubated at 37 °C for 30 min and stopped by addition of EDTA to 50 mM. In all reactions, less than 0.01 pmol of nucleotide was incorporated per picomole of 3′-OH termini, ensuring that the elongation of each reacted primer was the result of a single binding event of the polymerase (Wierowski et al., 1983). Following termination of the reaction, SDS (0.5%)–proteinase K (100 μg/mL) digestion of the reaction was carried out at 37 °C for 90 min. Samples were then twice extracted with phenol–chloroform–isoamyl alcohol (50:49:1) and once with chloroform–isoamyl alcohol (24:1). The aqueous phase was then filtered over Sephadex G-50 (Pharmacia) to remove unincorporated nucleotides. To ensure complete hydrolysis of the oligoribonucleotide primer before sizing, the purified DNA samples were boiled for 15 min in 0.2 N NaOH and further incubated at 37 °C for 2 h (Wierowski et al., 1983). Length determination of the products synthesized was performed as described by Fay et al. (1981). In brief, the alkaline-hydrolyzed sample was fractionated on an alkaline Bio-Gel A-5m (Bio-Rad) column (0.7 × 28 cm) equilibrated at room temperature with 5 mM EDTA and 0.1 N NaOH. Fractions (0.25 mL) were collected in 2-mL glass scintillation vials (Rochester Scientific, Inc.). Each frction was counted in Liquiscint as described above. Oligo(dT) standards (P-L Biochemicals) and orange G (Kodak) were used to calibrate the separation according to nucleotide length.

Analysis of DNA Products Synthesized by Electrophoresis. Reactions were carried out as described above. However, following SDS–proteinase K treatment, phenol–chloroform extraction, and gel filtration to remove unincorporated [^{32}P]dATP, the sample was lyophilized and resuspended in 95% (v/v) deionized formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. After being heated at 100 °C for 5 min, the samples were fractionated by electrophoresis at 1000 V for approximately 3 h in a 7 M urea–8.0% polyacrylamide gel (40 × 32 × 0.04 cm) containing 100 mM Tris–borate, pH 8.3, and 2 mM EDTA. Following electrophoresis, gels were autoradiographed with XAR-5 X-ray film (Kodak) at −70 °C with a Du Pont Lightning Plus intensifier screen.

Results

Homopolymeric Template Primers for Determining the Processivity of Nuclear Matrix Bound DNA Polymerase α. We have measured the processivity of nucleotide polymeri-

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DDT, dithiothreitol; AMPPNP, 5′-adenylyl imidodiphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); AMPPCP, 5′-adenylyl methylenediphosphate; Ap_4A, P^1,P^4-bis(5′-adenosyl) tetraphosphate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

Table I: Activity of Nuclear Matrix Bound DNA Polymerase on Exogenous Templates[a]

exogenous template	% relative activity
activated calf thymus DNA	100
poly(dT)·oligo$(A)_{10}$	59.6
poly(dA)·oligo$(dT)_{15}$	29.9
poly(dC)·oligo$(G)_{19-14}$	13.7
poly(dC)·oligo$(dG)_{10}$	2.1
poly(dA)·oligo$(U)_{19-24}$	1.9
poly(dT)·oligo$(dA)_{19-24}$	1.8
poly(A)·oligo$(dT)_{15}$	2.3

[a] Nuclear matrix was prepared from regenerating rat liver and assayed for DNA polymerase activities with exogenous templates as described under Experimental Procedures. Values are expressed as the percent activity relative to the activity with activated calf thymus DNA as template (24.8 pmol of dNMP incorporated per reaction) and represent the exogenous activities minus endogenous incorporation with one dNTP for homopolynucleotide templates and four dNTPs for calf thymus DNA template. The average primer gap size was 10 nucleotides for all homopolymer templates.

Table II: Activity of Nuclear Matrix Bound DNA Polymerase with Poly(dT)·Oligo$(A)_{10}$ as Template Primer[a]

reaction conditions	% relative activity
complete	100
complete − poly(dT)·oligo$(A)_{10}$	4.0
complete + 10 mM *N*-ethylmaleimide[b]	4.8
primer gap size of poly(dT)·oligo$(A)_{10}$	
100 nucleotides	85.5
250 nucleotides	58.5

[a] Nuclear matrix prepared from regenerating rat liver was assayed for DNA polymerase activity with poly(dT)·oligo$(A)_{10}$ as exogenous template (see Experimental Procedures). The assays were performed with an average primer gap size of 10 nucleotides unless indicated otherwise. [b] Preincubation with sample at 0 °C for 30 min.

zation by nuclear matrix bound DNA polymerase α using the product size method first reported by Fay et al. (1981). In this techinque, defined homopolymeric template primers are used to determine the lengths of DNA products synthesized when primers of known length are extended after one binding of the DNA polymerase to each reacted primer terminus. The processively synthesized DNA products are then separated by gel filtration or gel electrophoresis (see Experimental Procedures for more details). The homopolymeric template primers that were most active on the matrix-bound polymerase were poly(dT)·oligo$(A)_{10}$, poly(dA)·oligo$(dT)_{15}$, and poly(dC)·oligo$(G)_{19-24}$ (Table I). The low activity obtained with poly(A)·oligo$(dT)_{15}$ was completely resistant to preincubation with 10 mM *N*-ethylmaleimide. This demonstrated the presence of a small amount of DNA polymerase β and the absence of the highly processive DNA polymerase γ in the total nuclear matrix bound polymerase activity (Scovassi et al., 1980; Yamaguchi et al., 1980). Moreover, monoclonal antibodies to human DNA polymerase α (SJK-287), which neutralize the activity of purified human DNA polymerase α (Tanaka et al., 1982), inhibited the matrix-bound α polymerase activity by >95% (data not shown).

We decided to study the properties of poly(dT)·oligo$(A)_{10}$ in more detail since it was the homopolymeric template primer most effectively utilized by the matrix-bound polymerase. One advantage of poly(dT)·oligo$(A)_{10}$ is that DNA polymerase β does not effectively utilize oligoribonucleotide-primed DNA templates (Chang & Bollum, 1972; Falaschi & Spadari, 1978), and therefore, contribution of β polymerase to the total nuclear matrix activity is negligible (Table II). Also, the use of only one deoxyribonucleotide in the assay greatly reduced the contribution of synthesis on the endogenous matrix-attached DNA to the total activity (Table II; Smith & Berezney, 1982).

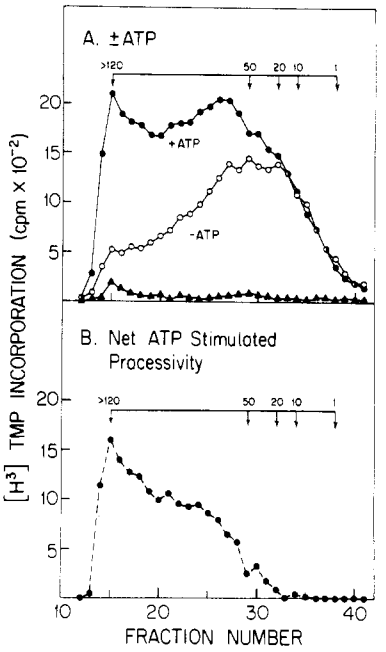

FIGURE 1: Gel filtration profiles of DNA products synthesized by nuclear matrix bound DNA polymerase α. The DNA products synthesized by nuclear matrix bound polymerase in the standard reaction for determining processivity were separated on an alkaline Bio-Gel A-5m column (see Experimental Procedures). Panel A shows the products synthesized with poly(dT)·oligo$(A)_{10}$ as exogenous template primer with (●) or without (○) 1 mM ATP and the endogenous DNA synthesis without poly(dT)·oligo$(A)_{10}$ in the reaction (▲). Panel B shows the calculated product size distribution for net ATP-stimulated activity and was obtained by subtracting the product size profiles in the absence of ATP from that in the presence of 1 mM ATP. Arrows indicate the nucleotide lengths corresponding to the elution positions of (from left to right) the void volume, $d(T)_{40-60}$, $d(T)_{20}$, $d(T)_{10}$, and orange G.

Thus, poly(dT)·oligo$(A)_{10}$ enabled us to directly study the processivity of the matrix-bound DNA polymerase α without significant interference from DNA polymerase β, or γ or from endogenous DNA synthesis. Furthermore, activity and processivity of the matrix-bound DNA polymerase α similar to those described in this paper with poly(dT)·oligo$(A)_{10}$ were also found when poly(dA)·oligo$(T)_{15}$, poly(dA)·oligo$(G)_{19-24}$, or single-stranded bacteriophage fd DNA with a known number of primers per molecule (Wierowski et al., 1983) was substituted as template primer.

Processivity of Nuclear Matrix Bound DNA Polymerase α and Its Enhancement by ATP. Figure 1A shows the size distribution of DNA products following DNA synthesis with nuclear matrix associated DNA polymerase. Under the reaction conditions employed (<0.01 pmol of nucleotide incorporated per picomole of available 3′-OH terminus), the size of each DNA tract represents the length of synthesis after one productive binding event between the polymerase and the DNA template (see Experimental Procedures). The majority of DNA products synthesized by the nuclear matrix bound DNA polymerase α ranged from 10 to 60 nucleotides. DNA tracts >60 nucleotides were found in progressively decreasing amounts including a small but reproducible fraction in the void volume (>120 nucleotides). The minimal average product size or processivity (minimal because products in the void volume are considered to be 120 nucleotides) was about 30 nucleotides (28.4 ± 5.2, $n = 3$). This value is severalfold higher than the average processivity reported for a number of purified DNA polymerase α preparations (5–15 nucleotides; Das & Fujimura, 1979; Fisher et al., 1979; Detera et al., 1981; Hockensmith & Bambara, 1981; Villani et al., 1981; Vierowski et al., 1983).

Moreover, purified preparations of DNA polymerase α typically demonstrate product size profiles that are less heterogeneous and essentially devoid of products >50 nucleotides (Wierowski et al., 1983).

We previously reported that ATP stimulated nuclear matrix bound DNA polymerase α activity (Smith & Berezney, 1982), and Wierowski et al. (1983) demonstrated that ATP enhanced both the activity and processivity of certain forms of purified calf thymus DNA polymerase α. Addition of 1 mM ATP increased the matrix-bound DNA polymerase activity an average of 2.3-fold (15 determinations) and resulted in a substantial increase in the proportion of products >50 nucleotides including a large increase in the void volume (Figure 1A). This raised the minimal mean product size from about 30 to 50 (48.7 ± 6.4, n = 3) nucleotides. Subtraction of the product size distribution in the absence of ATP from that in the presence of ATP enabled us to plot the product size distribution of net ATP-stimulated DNA polymerase α activity. As shown in Figure 1B, nearly all the products are greater than 50 nucleotides with a progressively increasing amount of radioactivity in longer products up to the void volume (>120 nucleotides). The minimal estimate of the average product size for the net ATP-stimulated activity shown in Figure 1B was 82.7 nucleotides and averaged 81.4 ± 4.3 for three separate preparations of regenerating rat liver nuclear matrix.

Processivity of DNA Polymerase α in Total Nuclei, High-Salt Extract, and Nuclear Matrix. Approximately 70–80% of the total nuclear DNA polymerase α activity is released into the high-salt extract during nuclear matrix preparation (Smith & Berezney, 1982). The average length of DNA snythesized by the salt-soluble polymerase (8.9 nucleotides) was severalfold less than the matrix-bound polymerase (Figure 2B,C). Also the size distribution profile of the salt-soluble polymerase was much less heterogeneous than the nuclear matrix profile with most of the DNA products being approximately 10 nucleotides and very few products ≥50 nucleotides (Figure 2B). Addition of ATP (1 mM) had no measurable effect on either activity or product size distribution (Figure 2B).

To determine whether the different processivity properties of the salt-soluble and matrix-bound DNA polymerases are native to the cell nucleus or created during nuclear matrix isolation, we measured the size distribution of DNA products synthesized in total cell nuclei. The DNA product profile of total nuclear DNA polymerase α had characteristics of both the salt-extractable and matrix-bound polymerases, respectively. Most of the DNA products were small (5–50 nucleotides) as in the high salt soluble profile, but there was also a significant shift to longer DNA products (≥70 nucleotides) including a small but reproducible amount in the void volume (Figure 2A).

Next we constructed a theoretical total nuclear product size distribution profile based on the salt-soluble and matrix-bound polymerase profiles of Figure 2B,C and a distribution of total nuclear DNA polymerase α activity in the salt-soluble and matrix fractions of 72% and 28%, respectively (see Figure 2 legend). The theoretical size distribution resembled the experimental profile of Figure 2A (data not shown) and had an average DNA length of 21.2 nucleotides compared to 22.4 nucleotides for Figure 2A. We also constructed a theoretical matrix profile by subtracting 78% of the high salt soluble size distribution profile (Figure 2B) from the corresonding total nuclear profile (Figure 2A). Again, the constructed DNA size distribution resembled the experimental matrix distribution profile of Figure 2C and had an average processivity of 54.8 nucleotides compared to 49.3 for Figure 2C (data not shown). In addition, there was no measurable degradation of ^{32}P-labeled poly(dT)·oligo(A)$_{10}$ when incubated in the DNA synthesis reactions with total nuclei, high-salt extract, or nuclear matrix. We, therefore, conclude that differential nuclease action is not a significant factor in the DNA product size measurements.

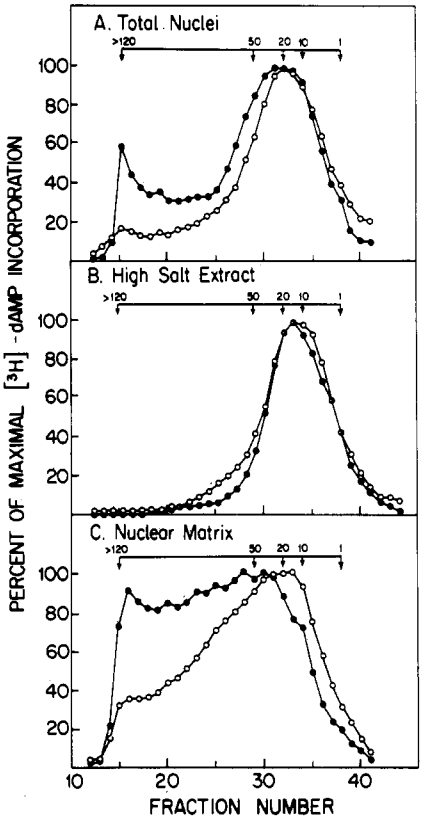

FIGURE 2: Gel filtration profiles of DNA products synthesized by DNA polymerase α in total nuclei, high-salt extract, and nuclear matrix. DNA synthesis under conditions for determining processivity with poly(dT)·oligo(A)$_{10}$ as template primer was performed in the presence (●) and absence (○) of 1 mM ATP, and the products were separated on an alkaline Bio-Gel A-5m column as described under Experimental Procedures. For direct comparison, all assays were performed in the absence of KCl at the same final NaCl concentration (50 mM) as the high-salt extract. This resulted in a slight decrease in total nuclear and nuclear matrix activity (<20%) but had no measurable effect on the product size distribution. Maximal incorporations correspond to 1287 cpm with ATP and 784 cpm without ATP for total nuclei (panel A), 4122 cpm with ATP and 4822 cpm without ATP for high-salt extract (panel B), and 2417 cpm with ATP and 2259 cpm without ATP for nuclear matrix (panel C). In this particular experiment, approximately 72% of the total nuclear DNA polymerase α activity was recovered in the high-salt extract (salt-soluble polymerase) with about 28% remaining associated with the salt-resistant nuclear matrix (see Experimental Procedures). DNA synthesized by polymerase β represented only a minor component of the profiles (<10%) as a determined by the N-ethylmaleimide sensitivity of synthesis (see Experimental Procedures).

Processivity of DNA Polymerase α Solubilized from Nuclear Matrix. In an attempt to determine the relationship of the nuclear matrix bound state of DNA polymerase α to the ATP stimulation of processivity and activity, we solubilized the polymerase from the nuclear matrix. Treatment of nuclear matrices with gentle sonication in the presence of 2 M NaCl resulted in the release of approximately 75% of total nuclear matrix bound DNA polymerase α activity into a nuclear matrix extract. Most of the remaining matrix-bound activity was recovered in the residual matrix pellet (see Experimental Procedures). A more detailed study of this fractionation and the properties of the matrix-released polymerase activities was

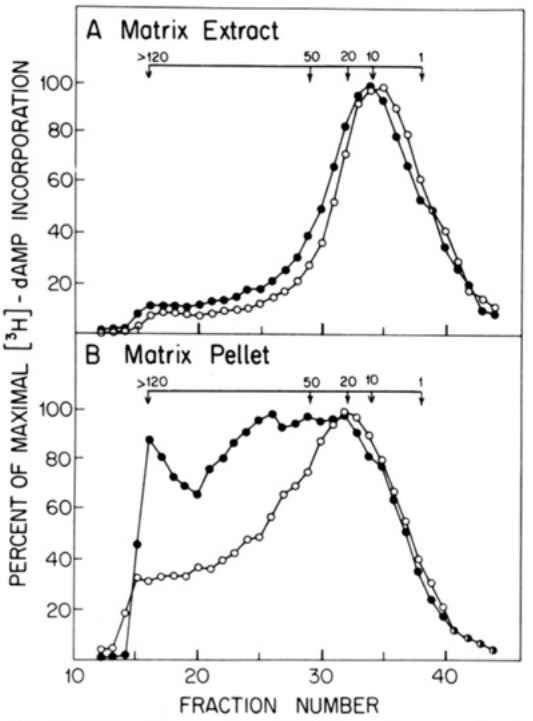

FIGURE 3: Gel filtration profiles of DNA products synthesized by DNA polymerase α from nuclear matrix extract and pellet. DNA polymerase α was solubilized from nuclear matrix into a matrix extract (70–80% of total matrix-bound activity) while the residual matrix-bound activity (20–30%) was recovered in a low-speed matrix pellet (see Experimental Procedures). Reactions with poly(dT)·oligo$(A)_{10}$ as template primer under conditions for measuring the processivity of DNA synthesis in the presence (●) and absence (○) of 1 mM ATP and separation of the DNA products on an alkaline Bio-Gel A-5m column were performed as described under Experimental Procedures. All assays were performed in the absence of KCl at the same final NaCl concentration (50 mM) as the nuclear matrix extract. This only slightly decreased activity (<20%) and had no measurable effect on the product size distribution of the nuclear matrix bound polymerase. Maximal incorporation corresponds to 3004 cpm with ATP and 4285 cpm without ATP for matrix extract (panel A) and 2085 cpm with ATP and 1865 cpm without ATP for matrix pellet (panel B).

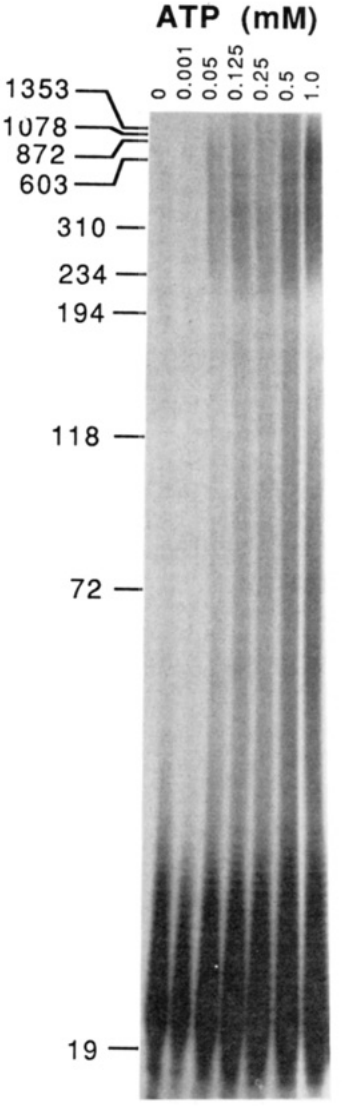

FIGURE 4: Effect of ATP concentration on size of products synthesized processively by nuclear matrix bound DNA polymerase α. Primers were extended on poly(dT)·oligo$(A)_{10}$ in the presence of increasing ATP concentration under conditions for measuring processive DNA synthesis (see Experimental Procedures). Results are presented as an autoradiogram of ^{32}P-labeled DNA products run on a DNA sequencing gel (see Experimental Procedures). Approximately equal cpm were added to each lane of the gel. Markers for nucleotide length were generated from *Hin*dIII-restricted γ DNA.

recently published (Tubo & Berezney, 1987b).

The release of matrix-bound DNA polymerase α resulted in a large decrease in the average processivity from approximately 50 to 6.8 nucleotides (Figure 3A) with no measurable effect of ATP (1 mM) on either activity or processivity. A relatively homogeneous population of DNA tracts was detected from about 5 to 20 nucleotides with very few products exceeding 50 nucleotides. Noticeably absent was the heterodisperse population of DNA products from about 60 to >120 nucleotides, which is characteristic of the nuclear matrix bound DNA polymerase α (Figure 1A and 2C).

In contrast to the matrix-solubilized activity, the DNA polymerase α remaining in the matrix structure following sonication (20–30% of total activity) was stimulated 2–3-fold by 1 mM ATP and had a product size profile and average processivity virtually indistinguishable from the total matrix-bound activity. This suggests that the mild sonication, per se, does not indiscriminately destroy proessive synthesis and that at least some aspect of the matrix-bound state of α polymerase is essential for ATP-stimulated, highly processive synthesis of DNA. Moreover, incubation with ^{32}P-labeled poly(dT)·oligo$(A)_{10}$ during the DNA synthesis reactions suggested that the large decrease in the processivity of DNA synthesis following release of the matrix-bound DNA polymerase α was not due to the activation of a cryptic nuclease activity following the sonication step. Attempts to reconstitute the ATP-enhanced processivity have been complicated so far by the inability to rebind the released enzyme to the nuclear matrix structure (R. A. Tubo and R. Berezney, unpublished experiments).

Nucleotide Requirements for Stimulation of Nuclear Matrix Bound DNA Polymerase α Activity and Enhancement of Processive Synthesis. Since a considerable amount of the DNA synthesized by the nuclear matrix bound DNA polymerase α in the presence of 1 mM ATP was recovered in the void volume (>120 nucleotides) following separation on Bio-Gel A-5m columns (see Figure 1), we decided to size the DNA products on 8% polyacrylamide–7M urea sequencing gels (see Experimental Procedures). Figure 4 shows an autoradiogram in which the DNA products of processive synthesis with poly(dT)·oligo$(A)_{10}$ as template primer and [α-^{32}P]dATP as the radioactive nucleotide are displayed at different concentrations of ATP. Concentrations of 1–10 μM ATP did not significantly shift the product size distribution from reactions

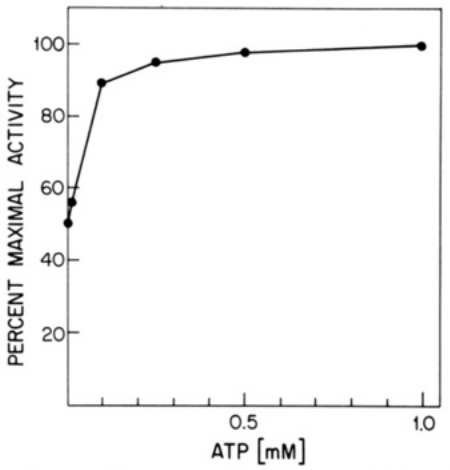

FIGURE 5: Effect of ATP on nuclear matrix bound DNA polymerase α activity. Nuclear matrices were assayed for in vitro DNA synthesis in the presence of increasing ATP concentrations with poly(dT)·oligo$(A)_{10}$ as template primer (see Experimental Procedures). Results are expressed as the percent of maximal DNA polymerase activity vs. ATP concentration. Incorporation in the absence of ATP was 2.19 pmol of dAMP. Maximal incorporation (100%) at 1.0 M was 4.30 pmol of dAMP. At concentrations of ATP > 1 mM, the activity continued to increase gradually until 4 mM ATP. Most of the activity increase (~80% of maximal activity), however, was completed by 100 μM ATP. The results plotted represents the average of two separate experiments.

Table III: Effect of Nucleotides on Nuclear Matrix Bound DNA Polymerase Activity[a]

addition	x-fold stimulation	addition	x-fold stimulation
minus nucleotide	1.0	dGDP	2.7
ATP	3.1	dCDP	2.3
GTP	2.7	dTDP	2.5
UTP	2.7	ATPγS	2.8
CTP	2.6	AMPPNP	2.8
dGTP	2.5	AMPPCP	0.9
dTTP	2.5	ATPαS	2.6
dGTP	2.3	AMP	1.0
ADP	2.9	ADPβS	1.1
GDP	3.0	Ap_4A (10 μM)	0.9
UDP	2.7	Ap_4A (100 μM)	0.8
CDP	2.6	Ap_4A (1 mM)	0.6
dADP	2.7	pyrophosphate	0.9

[a] DNA polymerase was measured in nuclear matrix with poly(dT)·oligo$(A)_{10}$ as template (see Experimental Procedures). Incorporation of dAMP in the presence of various nucleotides or pyrophosphate (1 mM, unless otherwise indicated) was then divided by the incorporation in the standard assay minus added nucleotide to give the x-fold stimulation.

performed in the absence of ATP (Figure 4 and additional data). At 50 μM ATP there was significant increase in DNA tracts ≥50 nucleotides up to full template length (1000–1200 nucleotides; Figure 4). This was followed by a gradual increase in the proportion of longer DNA products (>50–1200 nucleotides) with increasing concentrations of ATP to 1 mM.

The ATP stimulation of nuclear matrix bound DNA polymerase α activity under the reaction conditions used for measuring the processive synthesis of DNA with poly(dT)·oligo$(A)_{10}$ as template primer is shown in Figure 5. An approximately 2-fold increase in activity was measured at 1 mM ATP. However, most of this increase (75–80%) was achieved by 100 μM ATP. Moreover, the activity did not reach a maximum at 1 mM ATP but gradually increased up to 4 mM ATP where the activity is about 3.0-fold higher than that in the absence of ATP (data not shown). It should be noted that the initial increase in polymerase activity by ATP

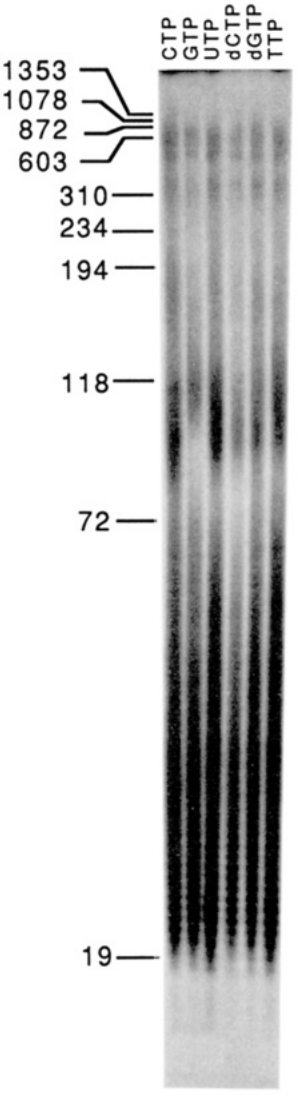

FIGURE 6: Effect of ribo- and deoxyribonucleoside triphosphates on size of products synthesized processively by nuclear matrix bound DNA polymerase α. Primers were extended on poly(dT)-oligo$(A)_{10}$ in the presence of 1 mM dNTP under conditions for measuring processive DNA synthesis (see Experimental Procedures). Results are presented as an autoradiogram of ^{32}P-labeled DNA products run on a DNA sequencing gel (see Experimental Procedures). Approximately equal cpm were applied to each lane of the gel. Markers for nucleotide length were generatedfrom *Hind*III-restricted λ DNA.

up to 1 mM correlates well with the increase in processivity, which is also largely completed by approximately 100 μM ATP (compare Figure 4 and 5). In contrast, the gradual but significant increase in activity between 1.0 and 4.0 mM ATP is not accompanied by any further shift to larger DNA products as detected by either Bio-Gel A-5m columns or sequencing gels (data not shown).

We next determined whether the stimulation of processivity and activity of the nuclear matrix bound DNA polymerase α was specific for ATP or whether other nucleotides could substitute for ATP. Sequencing gel electrophoretic analysis showed that each NTP or dNTP (1 mM) shifted the product size distribution up to full template length similar to ATP (Figure 6). Correlated with the enhancement of processivity was a 2–3-fold stimulation of activity (Table III). Further studies demonstrated that all the (d)NDP's also stimulated activity 2–3-fold at 1 mM concentrations (Table III) and elicited a similar shift to long DNA products (Figure 7B and data not shown). Like ATP, most of the activity and processivity increases were completed by 100 μM for each (d)NTP

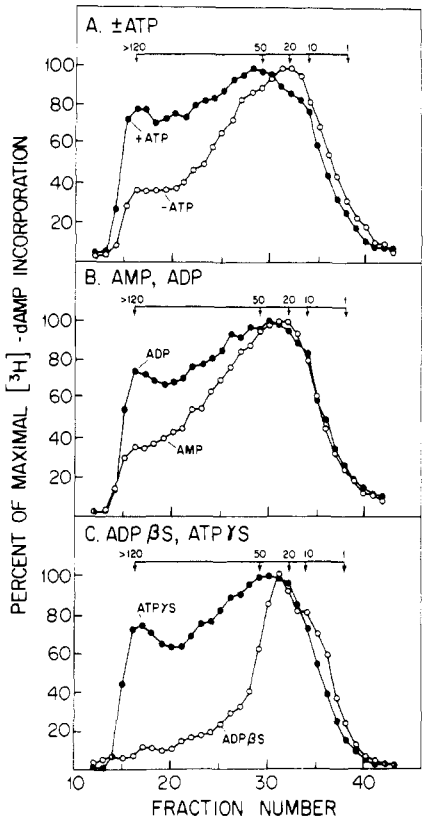

FIGURE 7: Effect of ADP, AMP, ATP, and ADP-nonhydrolyzable analogues on size of processively synthesized DNA products. The DNA products synthesized by nuclear matrix bound polymerase in the presence of various nucleotides (1 mM) with the standard reaction for determining processivity were separated on an alkaline Bio-Gel A-5m column (see Experimental Procedures). Maximal incorporations correspond to 1861 and 1387 cpm in the presence (●) and (○) absence of ATP (panel A), 1832 and 1492 cpm with ADP (●) and AMP (○), respectively (panel B), and 2334 and 2193 cpm with ATPγS (●) and ADPβS (○), respectively (panel C).

and (d)NDP investigated. In contrast, nucleoside monophosphates such as AMP had no effect on either the activity (Table III) or processivity (Figure 7B).

The possible correlation between activity and processivity stimulations for the nuclear matrix bound DNA polymerase α was further strengthened by analysis of several nonhydrolyzable analogues of ATP and ADP. Modifications at the α-phosphate group of ATP (ATPαS), γ-phosphate of ATP (ATPγS), or β,γ-phosphoanhydride linkage (AMPPNP) stimulated both activity (Table III) and processivity (Figure 7C and data not shown) to similar levels as ATP. In contrast, modifications at the β-phosphate group (ADPβS) resulted in no measurable stimulation of either activity (Table III) or processivity (Figure 7C).

Taken together these results suggest that the ATP enhancements of activity and processivity are closely related and apparently do not require the free energy of phosphoanhydride bond hydrolysis. Instead, an important role for the stereochemistry of the effective nucleotides is suggested and further supported by the lack of effects by pyrophosphate, AMPPCP, and the bulkier nucleotide Ap_4A on activity (Table III) or processivity (data not shown). Indeed, Ap_4A at 1 mM significantly inhibited DNA polymerase α activity (Table III).

Discussion

A number of recent findings suggest that in vivo DNA replication in eucaryotic cells is associated with a proteinaceous subnuclear structure termed the nuclear matrix (Berezney 1984, 1985; Carri et al., 1986; Dijkwel et al., 1986). The kinetics of association of in vivo replicating DNA (Berezney & Coffey, 1975, 1976; Dijkwel et al., 1979; McCready et al., 1980; Pardoll et al., 1980; Berezney & Buchholtz, 1981b) with isolated nuclear matrix have lead to a new view of eucaryotic replication in which it is proposed that DNA loops corresponding to replicon subunits (Huberman & Riggs, 1968; Hand, 1978) are replicated by translocation across nuclear matrix bound replisome assemblies. The replicative-dependent association of DNA polymerase α, primase, and other replicative enzymes with the nuclear matrix (Smith & Berezney, 1980, 1982, 1983; Foster & Collins, 1985; Tubo et al., 1985; Wood & Collins, 1986; Tubo & Berezney, 1987) has provided the first enzymatic evidence to support this nuclear matrix replisome model.

These previous studies have stimulated us to investigate the translocation of DNA during in vitro DNA synthesis in regenerating rat liver nuclear matrix. Translocation of a polymerase relative to the DNA template during DNA synthesis can be estimated by measuring the processivity of the enzyme, which is defined as the number of nucleotides added during a single, productive binding event of the polymerase to the DNA template (Bambara et al., 1978; Fay et al., 1981; Hockensmith & Bambara, 1981; Wierowski et al., 1983). It has been suggested (Hockensmith & Bambara, 1981) that the processivity of DNA polymerase α at native in vivo replicational forks is likely to approach at least the length of Okazaki fragments (100–300 nucleotides). Processivities of purified forms of DNA polymerase α, however, are characteristically low (e.g., 5–15 nucleotides; Das & Fujimura, 1979; Fisher et al., 1979; Detera et al., 1981; Hockensmith & Bambara, 1981; Villani et al., 1981; Wierowski et al., 1983). The addition of ATP, other nucleotides, and various protein subunits lost during purification of DNA polymerase α significantly increases the processivity of the enzyme, perhaps recreating more native conditions (Hockensmith & Bambara, 1981; Wierowski et al., 1983).

In this paper we demonstrate that the average mean processivity of the nuclear matrix bound DNA polymerase α is severalfold higher ($\sim$30 nucleotides) than typical preparations of purified α polymerase (Figure 1A). Moreover, the activity and processivity of the matrix-bound polymerase is markedly enhanced by ATP (1 mM). Evaluation of the DNA products from the net ATP-stimulated activity indicated that the increased activity was due almost exclusively to processively synthesized products of 50 to >120 nucleotides with a minimal mean average processivity exceeding 80 nucleotides (Figure 1B). Sizing of the DNA products on urea–acrylamide sequencing-type gels revealed that the products >120 nucleotides ranged up to full template length (1000–1200 nucleotides; Figure 4). These experiments also demonstrated that the enhancement of both the activity and processivity of matrix-bound DNA polymerase was largely completed by 100 μM ATP (Figures 4 and 5). While this exquisite sensitivity to ATP appears to contrast with the much higher ATP concentrations (4 mM) reported to be optimal for purified preparations of polymerase (Wierowski et al., 1983; Lawton et al., 1984), it should be noted that the activity of the matrix-bound polymerase continues to increase, albeit slowly, as the ATP increases from 1 to 4 mM but with no further enhancement in processivity (data not shown).

Unlike the results reported by Wierowski et al. (1983) and Lawton et al. (1984) for certain soluble forms of DNA polymerase α, the ATP stimulation of nuclear matrix bound DNA polymerase α does not appear to require the hydrolysis

of a high-energy phosphoanhydride linkage. All ribo- and deoxyribonucleoside di- and triphosphates stimulated activity and processivity to a similar level as ATP at both 1 mM (Table III and Figures 5 and 7) and 100 μM (data not shown). Moreover, the nonhydrolyzable ATP analogues AMPPNP, ATPγS, and ATPαS were equally effective while the nucleoside monophosphates such as AMP (Table III and Figure 7) did not enhance either activity or processivity. A certain degree of specificity in nucleotide stimulation was indicated by the inability of the ADP-nonhydrolyzable analogue ADPβS to stimulate activity or processivity (Table III and Figure 7). This suggests a critical role of the β-phosphate moiety of this nucleotide for activation. The enhancement, however, is likely to involve more than the β-phosphate group, since neither pyrophosphate nor the ATP analogue AMPPCP (which has an intact β-phosphate group but a considerable change in the P–C–P bond angles and distances compared to P–O–P; Yount et al., 1971) was stimulatory. Additionally, diadenosine tetraphosphate (Ap_4A), which has an intact β-phosphate group but two adenine moieties and is a much bulkier molecule than ADP or ATP, significantly inhibited activity at 1 mM. To our initial surprise, Lindenbaum et al. (1986) have recently shown a very similar pattern of nucleotide activation including the lack of stimulation by ADPβS and stimulation by ATPγS and AMPPNP for the complex of the adenovirus-encoded 140-kDa DNA polymerase (Ad Pol) and the 59-kDa DNA binding protein (Ad DBP). Since the Ad Pol–Ad DBP complex can synthesize adenoviral DNA with a remarkable degree of processivity (Field et al., 1984), this leads us to consider that the similar stimulatory patterns of the Ad Pol–Ad DBP complex and the matrix-bound DNA polymerase α may be characteristic of highly processive forms of DNA polymerase in eucaryotic cells.

Unlike the matrix-bound DNA polymerase, the approximately 70% of total nuclear DNA polymerase α that was extracted from isolated nuclei by salt had a much lower processivity (average of nine nucleotides), and neither the activity nor the processivity was stimulated by 1 mM ATP (Figure 2B). The lower processivity of the salt-soluble enzyme is not a consequence of the salt extraction since total nuclear DNA polymerase α showed a product size distribution consistent with the individual profiles of the high salt soluble and matrix-bound profiles (Figure 2A). Indeed, a theoretical nuclear product size distribution based on the high-salt and matrix-bound profiles was similar to the actual product size distribution in nuclei (data not shown). Appropriate controls also demonstrated that the differences in DNA size distributions among the nuclear fractions were not due to endogenous nuclease action. Our results, therefore, demonstrate that a discrete portion of the total nuclear DNA polymerase α (~30%) has a much higher processivity than the bulk nuclear polymerase. Moreover, this ATP-stimulated, higher processivity form of the total nuclear polymerase is exclusively associated with the isolated nuclear matrix.

Solubilization of the majority of the matrix-bound DNA polymerase α by sonication (see Experimental Procedures) resulted in a striking decrease in the average mean processivity of the matrix-solubilized DNA polymerase α (ca. seven nucleotides, Figure 3A) and no stimulation of activity or processivity by 1 mM ATP. In contrast, the residual DNA polymerase α (20–30% of total activity) that remained bound to the matrix fraction following sonication displayed a product size distribution and ATP enhancement that were indistinguishable from the total nuclear matrix bound activity (Figure 3B).

While the actual role(s) of the nuclear matrix in DNA replication remain(s) to be conclusively established, the results of our study suggest an involvement in the processivity of DNA synthesis. We propose that the matrix-bound DNA polymerase α is associated with certain protein subunits or other matrix-bound factors, which convey nucleoside di- or triphosphate dependent, highly processive synthesis. Upon dissociation of the polymerase from the nuclear matrix, these associations may become altered, changing the polymerase to a low-processivity form with characteristics of salt-soluble polymerase (Das & Fujimura, 1979; Fisher et al., 1979; Detera et al., 1981; Hockensmith & Bambara, 1981; Villani et al., 1981; Wierowski et al., 1983; this study). Investigations are under way to identify these putative matrix-bound processivity factors.

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Registry No. ATP, 56-65-5; ADP, 58-64-0; ATPγS, 35094-46-3; GTP, 86-01-1; UTP, 63-39-8; CTP, 65-47-4; dGTP, 2564-35-4; dTTP, 365-08-2; dCTP, 2056-98-6; GDP, 146-91-8; UDP, 58-98-0; CDP, 63-38-7; dADP, 2793-06-8; dGDP, 3493-09-2; dCDP, 800-73-7; dTDP, 491-97-4; AMPPNP, 25612-73-1; ATPαS, 29220-54-0; AMP, 61-19-8; ADPβS, 35094-45-2; DNA polymerase, 9012-90-2.

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