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Synthesis and Distribution of Primer RNA in Nuclei of CCRF-CEM Leukemia Cells†

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ABSTRACT: The distribution of primer RNA and RNA-primed nascent DNA in nuclei of CCRF-CEM leukemia cells was examined, and the primer RNA purified from the nuclear matrices of these cells was characterized. RNA-primed nascent DNA was radiolabeled by incubating whole-cell lysates with [α-^{32}P]ATP and [^{3}H]dTTP in the presence of approximately physiological concentrations of the remaining ribo- and deoxyribonucleoside triphosphates. The primer RNA was purified by cesium chloride density gradient centrifugation and analyzed by polyacrylamide gel electrophoresis. Nuclear subfractionation studies revealed that at least 94% of the primer RNA and RNA-primed nascent DNA were located within the insoluble matrix fraction of the nucleus. The predominant primer RNA isolated from the nuclear matrix was 8-10 nucleotides in length, and several lines of evidence indicated that this oligoribonucleotide was the functional primer RNA. Essentially all of the matrix primer RNA was covalently linked to the newly replicated DNA as demonstrated by its buoyant density in cesium chloride gradients, phosphate-transfer analysis, and sensitivity to DNase I. Analysis of ^{32}P transfer from [α-^{32}P]dTTP revealed a random distribution of ribonucleotides at the 3′-end of the primer RNA. Data obtained from mixing experiments indicated that the association of RNA-primed nascent DNA with the nuclear matrix was not the result of aggregation of these fragments with the nuclear matrix. No significant amount of either primer RNA, RNA-primed nascent DNA, or phosphate transfer was detected in the high-salt-soluble (nonmatrix) fraction of the nucleus, although the nonmatrix fraction contained most of the newly replicated DNA. These observations provide evidence that in CCRF-CEM cells the synthesis of both primer RNA and RNA-primed Okazaki fragments takes place on the nuclear matrix, and that the primer RNA is degraded prior to the migration of the nascent DNA away from the matrix-bound DNA replication sites.

The discontinuous synthesis of DNA (Okazaki) fragments on the lagging strand of the replication fork is an important biochemical process involved in DNA replication. Most of the Okazaki fragments that are recovered from eukaryotic cells contain on oligoribonucleotide primer that is covalently attached to the 5′-end of the fragment (Waqar & Huberman, 1975a,b; Tseng & Goulian, 1977; Tseng et al., 1979; Kitani et al., 1984). This primer RNA, which is about 10 nucleotides in length (Tseng & Goulian, 1977; Tseng et al., 1979; Kitani

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et al., 1984), provides the free 3′-hydroxy terminus required by the DNA polymerase α-primase complex for the initiation of Okazaki fragment synthesis. The DNA polymerase α-primase complex purified from mammalian cells usually consists of four polypeptides that range in mass from approximately 50 to 180 kDa, the smallest two of which contain the DNA primase activity (Hirose et al., 1988; Nasheuer & Grosse, 1988).

Results from recent studies suggest that the functional DNA primase activity is tightly bound to the nuclear matrix, the mainly proteinaceous framework that remains after salt and detergent extraction and deoxyribonuclease treatment of isolated nuclei. The activities of some of the enzymes involved in DNA replication, including DNA primase and DNA polymerase α, become markedly increased on the nuclear matrix just prior to the entry of cells into the DNA synthetic (S) phase (Foster & Collins, 1985; Tubo & Berezney, 1987a,b; Collins & Chu, 1988; Hirose et al., 1988). Usually between 20 and 30% of the total nuclear DNA primase is found to be tightly associated with the nuclear matrix of rapidly proliferating mammalian cells (Tubo & Berezney, 1987a; Hirose et al., 1988), although estimates between 72 and 90% have been reported (Wood & Collins, 1986; Collins & Chu, 1988). Tubo and Berezney described the synthesis of oligoribonucleotide products of 8–50 nucleotides by nuclear matrix preparations that had been incubated with either ATP or GTP (Tubo & Berezney, 1987c). The above observations raised the following questions. Is functional primer RNA of normal and discrete length (about 10 nucleotides) associated with the nuclear matrix following incubation of whole-cell lysates with the four ribo- and deoxyribonucleoside triphosphates? What is the distribution of primer RNA within whole nuclei, and is this primer RNA enriched in the insoluble matrix fraction of the nucleus? Since primer RNA is associated exclusively with DNA replication forks, then addressing the above questions should be a specific approach for evaluating the importance of the nuclear matrix in DNA replication. The striking localization of primer RNA and RNA-primed DNA observed on the nuclear matrix is consistent with the concept that the nuclear matrix is the subnuclear site of primer RNA synthesis and degradation in CCRF-CEM leukemia cells.

Experimental Procedures

Materials. The human CCRF-CEM leukemia cell line was provided by Dr. Joseph R. Bertino of the Memorial Sloan-Kettering Cancer Center and was propagated at 37 °C under 95% air–5% CO_2 in Fischer's medium supplemented with 10% heat-inactivated horse serum, penicillin (20000 units/L), and streptomycin (20 mg/L). Tissue culture medium, serum, and antibiotics were obtained from Grand Island Biological Co. Cells were checked periodically for *Mycoplasma* contamination with the Gen-Probe *Mycoplasma* ribosomal RNA hybridization kit obtained from Fisher Scientific Co. Dithiothreitol, phosphoenolpyruvate, actinomycin D, α-amanitin, poly(dT), and all of the ribonucleoside monophosphates and ribo- and deoxyribonucleoside triphosphates were purchased from Sigma Chemical Co. RNase-free pancreatic DNase I with a specific activity of 2200 Kunitz units/mg of protein was purchased from Worthington Biochemical Corp. Endonuclease-free DNA polymerase I (Klenow fragment) was obtained from Boehringer Mannheim Biochemicals. Nuclease-free proteinase K and nuclease-free cesium chloride were obtained from E. Merck Darmstadt and Fisher Scientific Co., respectively. Yeast tRNA and redistilled nucleic acid grade phenol were purchased from Bethesda Research Laboratories. Ultrapure urea was purchased from Schwartz/Mann Biotech. Oligoribonucleotide and oligodeoxyribonucleotide size markers and polynucleotide kinase were purchased from Pharmacia LKB Biotechnology, Inc. All oligonucleotide size markers were end-labeled with [γ-^{32}P]ATP and 10 units of polynucleotide kinase as described previously (Maniatis et al., 1982). RNasin ribonuclease inhibitor was obtained from Promega. [α-^{32}P]-ATP, [γ-^{32}P]ATP, and [α-^{32}P]dTTP with specific radioactivities of approximately 3000 Ci/mmol, and [*methyl*-^{3}H]-dTTP and [8-^{3}H]dATP with specific radioactivities of 66 and 12 Ci/mmol, respectively, were purchased from ICN Radiochemicals. Low-salt buffer consisted of 10 mM Tris-HCl (pH 7), 1 mM $MgCl_2$, 10 mM NaCl, and 1 mM PMSF.[1] High-salt buffer (1.5 M) consisted of 10 mM Tris-HCl (pH 7), 0.6 mM $MgCl_2$, 1.5 M NaCl, and 1 mM PMSF. High-salt buffer (3 M) consisted of 10 mM Tris-HCl (pH 7), 0.2 mM $MgCl_2$, 3 M NaCl, and 1 mM PMSF. TE buffer consisted of 10 mM Tris-HCl (pH 7.8) and 2 mM EDTA.

Radiolabeling of Newly Replicated DNA and RNA. Exponentially growing CCRF-CEM cells (1.6×10^8) were harvested by centrifugation at 4 °C, and the pellets were washed at 4 °C with a solution of 0.14 M NaCl and 0.01 M KPO_4 (pH 7). The pellet was resuspended in 8 mL of ice-cold 10 mM Tris-HCl (pH 7) buffer that contained 1 mM $MgCl_2$ and 1 mM PMSF. The cells were allowed to swell on ice for 15 min and disrupted with 25 strokes in a Dounce homogenizer. The whole-cell lysate of 1.6×10^8 cells was mixed with 2 mL of incorporation buffer to yield final concentrations of 40 mM NaCl, 5 mM $MgCl_2$, 50 mM sucrose, 30 mM Hepes, 0.4 mM $CaCl_2$, 1 μM [α-^{32}P]ATP, 2 μM [^{3}H]dTTP (final specific radioactivities 23 and 6.4 Ci/mmol, respectively), 100 μM of the three remaining deoxyribonucleoside triphosphates, 1 mM of the three remaining ribonucleoside triphosphates, 5 mM phosphoenolpyruvate, and 0.8 mM dithiothreitol at a final pH of 7.8. The whole-cell lysate was incubated at 25 °C for 10 min and then layered over 45% (w/v) sucrose. The nuclei were purified by centrifugation at 1900*g*, 4 °C, for 30 min. The purification was monitored by Geimsa staining. Two hundred structures were counted; 95% were intact nuclei with well-defined borders free of visible cytoplasmic contamination, 4% were whole cells, and 1% were intact nuclei that contained cytoplasmic tags. No significant loss of newly replicated [^{3}H]DNA from the nuclei occurred during the isolation procedure, since greater than 99% of the total [^{3}H]DNA present in these cells was recovered in the nuclear fraction.

Isolation of Newly Replicated DNA and RNA from the Matrix and High-Salt-Soluble Fractions of the Nucleus. The procedure followed for the isolation of the nuclear matrix was a modification of that previously developed in this laboratory (Fernandes et al., 1988). All procedures were carried out under RNase-free conditions. Nuclei that were purified as described above were gently resuspended in 8 mL of low-salt buffer at 4 °C. Eight milliliters of 3 M high-salt buffer was added to the suspension over a period of 1 h with gentle mixing to yield a final concentration of 1.5 M NaCl. The sample was incubated for an additional 30 min on ice and then placed in a water bath at 37 °C. The DNA was digested by the addition of 2000 units of RNase-free pancreatic DNase I for 30 min. The sample was centrifuged, and a 3-mL aliquot of the supernatant (nonmatrix fraction) was diluted with an equal volume of 20 mM Tris-HCl (pH 8) buffer containing 0.4% (w/v) SDS, 40 mM EDTA, and 800 μg/mL proteinase K.

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; ATP, adenosine 5′-triphosphate; dTTP, thymidine 5′-triphosphate.

The pellet (nuclear matrix) was washed once with 1 mL of 1.5 M high-salt buffer, once with low-salt buffer containing 1% (v/v) Triton X-100, and once with low-salt buffer and then resuspended in 10 mM Tris-HCl (pH 8) buffer containing 0.2% (w/v) SDS, 20 mM EDTA, and 200 μg/mL proteinase K. Both the matrix and nonmatrix fractions were incubated for 2 h at 60 °C. At the end of the incubation period, the nonmatrix fraction was diluted 5-fold with TE buffer, and sufficient quantities of sodium acetate and magnesium chloride were added to the matrix and nonmatrix fractions to yield final concentrations of 0.3 and 0.01 M, respectively. Each sample contained 12.5 μg of polyacrylamide and 13.5 μg of yeast tRNA as coprecipitant. The nucleic acids were precipitated overnight with 3 volumes of ethanol at 4 °C. The mixtures were centrifuged at 40000*g*, 4 °C, for 30 min, and the nucleic acids were purified from the pellets by quantitative extraction and back-extraction with TE buffer and phenol/chloroform as previously described (Maniatis et al., 1982). The recovery of newly replicated [^{3}H]DNA from the matrix and nonmatrix fraction was greater than 90%.

Cesium Chloride Density Gradient Centrifugation. Following phenol/chloroform extraction, aliquots of newly replicated DNA and RNA were removed from the nuclear matrix and nonmatrix samples. The aliquots were heated to 90 °C for 5 min, immediately cooled on ice, and then mixed with a cesium chloride solution to final density of 1.74 g/mL. The samples were centrifuged at 101000*g*, 20 °C, for 48 h in a Beckman 70.1 Ti rotor. Fractions of 0.45 mL each were eluted from the bottom of the tube, and the amount of radioactivity in an aliquot of each fraction was determined by liquid scintillation counting. No spillover of ^{32}P radioactivity into the ^{3}H channel or vice versa was observed following calibration of the liquid scintillation counter for dual-label counting. The densities of the solution along the cesium chloride gradient were determined by refractive index measurements of a blank gradient and subsequent conversion of the values to density by using a standard curve of cesium chloride solutions. RNA concentrations were determined by the orcinol reaction with pentose (Schneider, 1957).

Polyacrylamide Gel Electrophoresis. Fractions from various density regions of the cesium chloride gradients of the nuclear matrix and nonmatrix samples were pooled, and the nucleic acids were precipitated with ethanol as described above. The nucleic acids from each sample were then resuspended in 45 μL of 10 mM Tris-HCl (pH 7.8) that contained 1 unit/μL of RNasin and 1 mM dithiothreitol. Each sample was divided into three equal aliquots. To one of the aliquots, EDTA was added to a final concentration of 2 mM. $MgCl_2$, $CaCl_2$, and RNase-free pancreatic DNase I were added to the second aliquot to final concentrations of 1 mM, 0.1 mM, and 12 units/μL, respectively. To the third aliquot, sufficient KOH was added to yield a final concentration of 0.3 N. Each of the three samples from the matrix and nonmatrix fractions were then incubated for 22 h at 37 °C. Following the hydrolysis of the RNA in KOH, the DNA in the third aliquot was precipitated with perchloric acid and the supernatant was neutralized with KOH and retained (Waqar & Huberman, 1975). All of the samples were heated at 90 °C in 7 M urea for 5 min and then cooled on ice for 5 min to promote separation of any RNA/DNA hybrids. The samples were then electrophoresed in a 20% polyacrylamide–7 M urea gel as described previously (Maniatis et al., 1982). Oligonucleotide size markers consisted of [5′-^{32}P](A)$_3$, [5′-^{32}P](U)$_6$, [5′-^{32}P](A)$_8$, [5′-^{32}P]d(N)$_{20}$, and [5′-^{32}P]d(N)$_{25}$. Upon completion of the electrophoresis, the gel was covered with plastic wrap and autoradiography was carried out at −70 °C for 24 or 72 h using Kodak X-OMAT AR film and a Du Pont Lightning Plus intensifying screen.

Phosphate-Transfer Analysis. Exponentially growing CCRF-CEM cells were harvested and whole-cell lysates prepared as described above. The whole-cell lysate of 1.6×10^8 cells was mixed with 8 mL of incorporation buffer to yield final concentrations of 40 mM NaCl, 5 mM $MgCl_2$, 50 mM sucrose, 30 mM Hepes, 0.4 mM $CaCl_2$, 63 nM [α-^{32}P]dTTP (final specific activity 3000 Ci/mmol), 125 nM [^{3}H]dTTP (final specific activity 53 Ci/mmol), 1 mM each of ATP, GTP, CTP, and UTP, 5 mM phosphoenolpyruvate, and 0.8 mM dithiothreitol at a final pH of 7.8. The whole-cell lysate was incubated at 25 °C for 10 min, and then the nuclear matrices were prepared. Aliquots of the matrix and nonmatrix fractions that contained equal amounts of total RNA (76 μg) were removed, and the nucleic acids were precipitated with ethanol and purified by phenol/chloroform extraction as described above. Sufficient KOH was added to each sample to yield a final concentration of 0.05 M, and then the samples were heated at 100 °C for 45 min (Bock, 1967). Following hydrolysis of the RNA to ribonucleoside 2′(3′)-monophosphates, the DNA was precipitated with perchloric acid, and the supernatant was neutralized with KOH as described above. The hydrolysis products in the supernatant were then separated by reversed-phase HPLC on a μBondapak octadecylsilica column (3.9 mm × 30 cm). The column was eluted at a flow rate of 0.8 mL/min with a linear gradient of 0–10% (v/v) acetonitrile in 0.02 M sodium phosphate (pH 5.2) that was developed over 40 min. The four ribonucleoside 2′(3′)-monophosphate standards were coinjected with each sample and their elution positions determined by absorbance at 254 nm. Fractions of 0.4 mL each were collected, and the ^{32}P was quantitated by liquid scintillation counting.

Mixing Experiment. Whole-cell lysates were prepared from exponentially growing CCRF-CEM cells and incubated for 10 min at 25 °C with [α-^{32}P]ATP. Nuclei were isolated and the suspension was divided into two aliquots. Total newly replicated [^{32}P]RNA was isolated from the nuclei by ethanol precipitation in the presence of 0.3 M sodium acetate (pH 5.7) and 0.01 M magnesium chloride. [^{32}P]RNA-primed nascent DNA was purified from the nuclear matrix by cesium chloride centrifugation and polyacrylamide gel electrophoresis as described above. The [^{32}P]RNA-primed nascent DNA, which migrated in the range of 11 to approximately 100 nucleotides in a 20% polyacrylamide–urea gel, was eluted from the gel with an International Biotechnologies electroeluter. In the mixing experiments, the purified [^{32}P]RNA and the [^{32}P]-RNA-primed nascent DNA in low-salt buffer were each added in triplicate to 2×10^7 unlabeled nuclei. Nuclear matrices were prepared from the unlabeled nuclei and then washed once with 1.5 M high-salt buffer and once with low-salt buffer. Nonspecific association of the exogenous [^{32}P]RNA and the [^{32}P]RNA-primed nascent DNA with the matrix and nonmatrix fractions of the nucleus was determined by counting the radioactivity present in these fractions. The results obtained in the mixing experiment were compared to the amounts of endogenous [^{32}P]RNA and [^{32}P]RNA-primed nascent DNA measured in the matrix and nonmatrix fractions of the nucleus following pulse labeling of whole-cell lysates with [α-^{32}P]ATP. Endogenous RNA-primed nascent DNA was quantitated as the sum of the ^{32}P cpm present in the DNA and intermediate regions (1.67–1.77 g/mL) of the cesium chloride gradients of the nuclear matrix and nonmatrix samples.

DNA Primase Assay. Nuclei were prepared from exponentially growing CCRF-CEM cells and purified by centrifugation through 45% (w/v) sucrose. Nuclear matrices were then isolated from aliquots of the purified nuclei as described above and washed once with 1.5 M high-salt buffer and once with low-salt buffer at 4 °C. The whole nuclei and nuclear matrices were then sonicated with one 15-s burst at 15 W by use of a Branson 200 sonifier equipped with a microtip. DNA primase activity was measured in a reaction coupled to *Escherichia coli* DNA polymerase I (Klenow fragment). The replication of poly(dT) by *E. coli* DNA polymerase I was dependent upon previous synthesis of an oligoriboadenylate primer by the DNA primase activity present in the sonicates of either whole nuclei or nuclear matrices. The reactions were carried out at 37 °C for 45 min in the presence of the sample (1–9 μg of protein), in 50 mM Tris-HCl (pH 8), 1 mM dithiothreitol, 8 mM magnesium chloride, 750 μg/mL heat-inactivated bovine serum albumin, 300 μM ATP, 100 μM poly(dT) (dTMP concentration), 25 μM [^{3}H]dATP (final specific radioactivity 0.1 Ci/mmol), and 0.33 unit of endonuclease-free *E. coli* DNA polymerase I (Klenow fragment) in a final volume of 50 μL. Protein concentrations were determined by the Coomassie blue staining method (Bradford, 1976).

Results

This laboratory has recently reported a partial characterization of the nuclear matrix from CCRF-CEM leukemia cells (Fernandes et al., 1988). Briefly, nuclear matrices that were prepared by high-salt extraction and pancreatic DNase I digestion of purified nuclei contained 1–5, 16, 10, and 37% of the total nuclear DNA, RNA, protein, and phospholipid, respectively. The matrix consisted of a residual framework of fibrils and granules that were connected to a peripheral lamina. This remnant structure was devoid of a nucleolus, nuclear envelope, and histones, but was enriched in M_r 60 000–90 000 proteins and newly replicated DNA (0.1–1.6 kilobases) compared to whole nuclei.

DNA Primase Activity in Nuclei and Nuclear Matrices. The activity of DNA primase was determined in sonicates of whole nuclei and nuclear matrices of exponentially growing CCRF-CEM leukemia cells. DNA primase activity was measured with poly(dT) as template in a reaction coupled to *E. coli* DNA polymerase I (Klenow fragment). The following evidence indicated that under these conditions the incorporation of [^{3}H]dATP into acid-insoluble product was directly dependent on the priming of the poly(dT) template by DNA primase activity present in the nuclear extract. Enzyme activity was linear with respect to the amount of sample protein present in the assay, and no significant enzyme activity was detected in the absence of either poly(dT), ATP, or nuclear extract. Moreover, the activity that was measured appeared distinct from other RNA polymerases. The combination of 50 μg/mL each of actinomycin D and α-amanitin, which decreased total RNA synthesis greater than 50%, had no effect on DNA primase activity. A significant fraction (22%) of the total nuclear DNA primase activity was tightly bound to the nuclear matrix (Table I). Repeated washings of the isolated matrices with high- and low-salt buffers did not decrease the activity of DNA primase per milligram of matrix protein, which indicated that this enzyme was tightly bound to the matrix. These results are very similar to those obtained with regenerating rat liver (Tubo & Berezney, 1987a,c) and chick embryo cells (Hirose et al., 1988) but differ from the results of Wood and Collins who reported that between 72 and 90% of the DNA primase activity in HeLa cell nuclei was specifically associated with the nuclear matrix (Wood & Collins, 1986; Collins & Chu, 1988). It was not possible to accurately measure DNA primase activity in the nonmatrix because of the high salt (1.5 M NaCl) and DNase I concentrations present in this fraction.

Table I: DNA Primase Activity in Nuclei and Nuclear Matrices[a]

cellular fraction	units/mg of protein	total units	% of total activity on matrix
nucleus	165 ± 60	356 ± 49	
nuclear matrix	341 ± 80[b]	77 ± 12	22

[a] Nuclei were prepared from exponentially growing CCRF-CEM cells and purified by centrifugation through 45% (w/v) sucrose. Nuclear matrices were isolated and then washed with 1.5 M high-salt buffer and low-salt buffer. The nuclei and nuclear matrices were then mildly sonicated, and DNA primase activity in the sonicates was measured with poly(dT) as template in a reaction coupled to *E. coli* DNA polymerase I. One unit of DNA primase activity is defined as the quantity of enzyme required to catalyze the formation of 1 pmol of acid-insoluble product/min. Results are the means of five determinations ± standard deviations. [b] $p < 0.01$ compared to the mean specific activity of DNA primase in the whole nucleus.

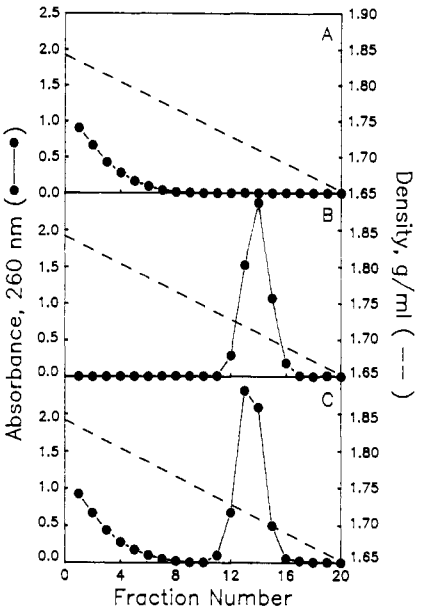

FIGURE 1: Cesium chloride density gradient centrifugation of RNA and DNA standards. Yeast tRNA and calf thymus DNA standards, either alone or in combination, were heated to 90 °C for 5 min, immediately cooled on ice, and then centrifuged to equilibrium in a cesium chloride gradient. Panel A, yeast tRNA alone; panel B, calf thymus DNA alone; panel C, mixture of yeast tRNA and calf thymus DNA.

Nuclear Distribution of RNA-Primed Nascent DNA. Since the nuclear matrix contained tightly bound DNA primase activity, it seemed reasonable that this remnant structure might likewise contain primer RNA and RNA-primed nascent DNA. Newly synthesized RNA and DNA were radiolabeled by incubating whole-cell lysates of exponentially growing CCRF-CEM cells for 10 min with [α-^{32}P]ATP and [^{3}H]dTTP. Samples of purified nucleic acids from the nuclear matrix and nonmatrix fractions, which each contained 76 μg of total RNA, were heat denatured in order to promote separation of any RNA/DNA hybrids. The denatured samples were then centrifuged to equilibrium in a cesium chloride density gradient. This centrifugation technique was capable of completely separating a yeast tRNA standard from a calf thymus DNA standard (Figure 1). If some of the ^{32}P-labeled primer was covalently attached to newly replicated [^{3}H]DNA, then this ^{32}P–^{3}H-labeled RNA-primed nascent DNA should band in

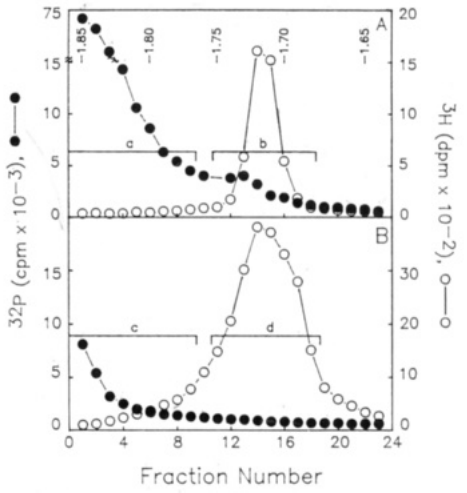

FIGURE 2: Cesium chloride equilibrium density gradient centrifugation of newly synthesized nucleic acids purified from the matrix (panel A) and high-salt-soluble (nonmatrix) (panel B) fractions of the nucleus. Newly synthesized RNA and DNA were radiolabeled by incubating whole-cell lysates of exponentially growing CCRF-CEM cells for 10 min at 25 °C with [α-^{32}P]ATP and [^{3}H]dTTP. Following heat denaturation, the purified nucleic acids were centrifuged in a cesium chloride density gradient as described in Figure 1. The upper region of panel A shows the densities in g/mL at various points along the gradients. The brackets in each panel show the fractions that were pooled for subsequent electrophoretic analysis.

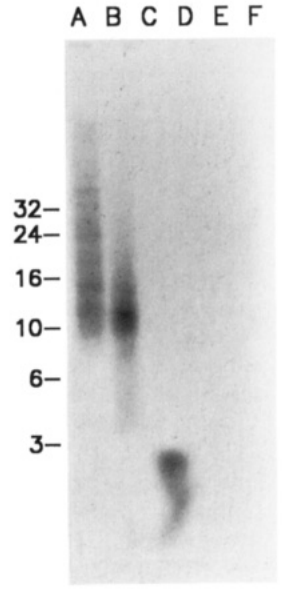

FIGURE 3: Autoradiogram of primer RNA and RNA-primed nascent DNA obtained from the DNA and intermediate density regions of the cesium chloride gradients. Newly replicated DNA and RNA were purified from the matrix and nonmatrix fractions of the nucleus, and aliquots of these fractions that contained equal amounts of total RNA were subjected to cesium chloride centrifugation. Fractions collected from the density region between 1.67 and 1.77 g/mL were pooled, and aliquots of the pooled samples were subjected to either no treatment, exhaustive DNase I digestion, or KOH hydrolysis. Following heat denaturation, the samples were electrophoresed on a 20% polyacrylamide–urea gel. Lanes A and D: Aliquots of the nuclear matrix and nonmatrix samples, respectively, that received no further treatment. Lanes B and E: Aliquots of the matrix and nonmatrix samples that were treated with DNase I prior to electrophoresis. Lanes C and F: Aliquots of the matrix and nonmatrix samples that were treated with KOH prior to electrophoresis. The autoradiographic exposure time of each lane was 72 h. Nucleotide size markers are shown to the left of lane A.

the cesium chloride gradient at a density less than that of the ^{32}P-labeled bulk RNA and nearer the DNA density region. Figure 2A shows that the nuclear matrix sample contained some ^{32}P-labeled RNA that migrated at an intermediate density (1.74–1.76 g/mL) between that of the newly synthesized RNA and DNA. In contrast to that seen after centrifugation of the matrix sample, a negligible amount of ^{32}P-labeled RNA from the high-salt-soluble fraction (nonmatrix) was present in this intermediate density region (Figure 2B). The lack of ^{32}P nonmatrix RNA in the intermediate density region of the cesium chloride gradient could not be attributable to a selective decrease in the recovery of nonmatrix RNA from the gradient. An average of 2.63×10^6 and 1.81×10^5 ^{32}P cpm from the matrix and nonmatrix samples was loaded on the respective gradients, and an average of 1.53×10^6 cpm (58 ± 13%) and 9.83×10^4 cpm (54 ± 2%), respectively, was recovered from the gradients. Since equal amounts of total RNA from the nuclear matrix and nonmatrix samples were subjected to density gradient centrifugation, the above data suggested that the insoluble matrix framework of the nucleus was enriched in RNA-primed Okazaki fragments relative to the high-salt-soluble fraction of the nucleus.

Characterization of Primer RNA and the RNA-Primed Nascent DNA. The isolation of RNA-primed nascent DNA was confirmed, and these intermediates characterized, by strand-separating polyacrylamide–urea gel electrophoresis and phosphate-transfer analysis. Full-length RNA-primed Okazaki fragments are about 200 nucleotides in length (Waqar & Huberman, 1975a,b; Tseng & Goulian, 1977; Tseng et al., 1979; Kitani et al., 1984). However, the total nuclear DNA was digested more than 97% by DNase I treatment during the preparation of the nuclear matrix. Thus, any RNA-primed nascent DNA that is isolated from the nuclear matrix should be less than 200 nucleotides in length and should be readily separable from the high molecular weight DNA and RNA by gel electrophoresis. Aliquots of the purified nucleic acids from the nuclear matrix and nonmatrix samples, which each contained 76 μg of total RNA, were centrifuged in a cesium chloride gradient as described above. Fractions were pooled from the DNA and intermediate density regions of the cesium chloride gradients as indicated by brackets b and d in Figure 2, and the nucleic acids were concentrated by ethanol precipitation. Samples consisting of the pooled nuclear matrix and nonmatrix fractions were each divided into three aliquots, heat denatured to separate any RNA/DNA hybrids, and then electrophoresed on a strand-separating 20% polyacrylamide–urea gel. As shown in Figure 3 (lane A), the ^{32}P label in the nuclear matrix sample migrated in the range of 9 to approximately 100 nucleotides. This represented the RNA-primed nascent DNA. The upper range was determined by extrapolation of the standard curve of nucleotide size markers. Lane B of Figure 3 shows the results obtained when an aliquot of the nuclear matrix sample was exhaustively digested with RNase-free pancreatic DNase I prior to electrophoresis in order to remove any DNA attached to the RNA. If the RNA was covalently linked to the DNA, then the DNase I digestion should result in an enhanced migration of the RNA. It can be seen in lane B that after exhaustive DNase I digestion the ^{32}P label became concentrated in a product of about 11 nucleotides in length, although lesser amounts of oligomers of 5–10 nucleotides were detected. This result is consistent with the isolation of intermediate and full-length primer RNAs. It cannot be explained by RNase contamination of the DNase I preparation because no ^{32}P-labeled nucleoside monophosphate product was seen in lane B. In addition, no digestion of a [5′-^{32}P](N)$_{3-8}$ oligoribonucleotide ladder was detected under identical incubation conditions (data not shown). Exhaustive DNase I digestion of RNA-primed nascent DNA has been shown to leave between 1 and 3 deoxyribonucleoside mono-

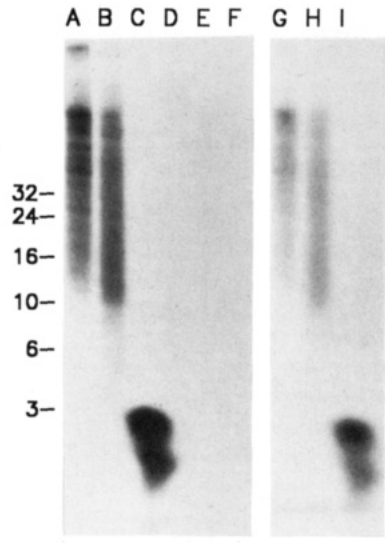

FIGURE 4: Autoradiogram of newly synthesized DNA and RNA obtained from the RNA density regions of the cesium chloride gradients. Fractions collected from the RNA density regions (1.78–1.85 g/mL) of the same cesium chloride gradient described in the legend to Figure 3 were electrophoresed on a 20% polyacrylamide–urea gel. Lanes A and D: Aliquots of the nuclear matrix and nonmatrix samples, respectively, that received no further treatment. Lanes B and E: Aliquots of the matrix and nonmatrix samples that were treated with DNase I prior to electrophoresis. Lanes C and F: Aliquots of the matrix and nonmatrix samples that were treated with KOH prior to electrophoresis. The autoradiographic exposure time of lanes A–F was 72 h. Lanes G, H, and I are the same samples shown in lanes A, B, and C, respectively, with the exception that the autoradiographic exposure time was reduced to 24 h.

phosphate residues attached to the 3′-end of the primer (Yamaguchi et al., 1985). Therefore, the actual length of the predominant primer RNA is estimated to be between 8 and 10 ribonucleotides. It was also reasoned that if the ^{32}P label was actually incorporated into the primer RNA portion of the RNA-primed nascent DNA, then the ^{32}P label should migrate as a mixture of ribonucleoside mono- and tetraphosphates following alkaline hydrolysis of the RNA-primed nascent DNA (Yamaguchi et al., 1985). The result shown in lane C of Figure 3 confirms this reasoning. Lanes D–F of Figure 3 show the results obtained following parallel treatments of the nonmatrix sample. Neither RNA-primed nascent DNA nor primer RNA could be detected in the nonmatrix samples by autoradiography of the electrophoresis gel.

It was possible that the nonmatrix sample contained primer RNA that was either (a) not extended sufficiently by DNA polymerase α to form Okazaki fragments or (b) no longer associated with DNA as a consequence of the DNase I treatment that was used in the preparation of the nuclear matrix. This primer RNA would remain undetected by the above procedure because the primer RNA would not have sedimented in the DNA density region of the preparative cesium chloride gradient. Therefore, the RNA density regions of the cesium chloride gradients (1.78–1.85 g/mL) depicted in Figure 2, panel A (bracket a) and panel B (bracket c), were also analyzed by polyacrylamide gel electrophoresis. No primer RNA was detected in the nonmatrix sample (Figure 4, lane E) obtained from the RNA density regions of the cesium chloride gradients. Although the size distribution of the [^{32}P]RNA from the matrix sample (Figure 4, lane B) was slightly smaller after exhaustive DNase I digestion, this digestion did not result in the concentration of the ^{32}P label in an RNA product of about 11 nucleotides in length as was seen with the matrix sample from the DNA density region (Figure 3, lane B). It might be argued that lanes B of Figures 3 and 4 are not qualitatively different and that Figure 4 is merely a more exposed version of Figure 3. In order to demonstrate that the [^{32}P]RNA present in lane B of Figures 3 and 4 are qualitatively different, the autoradiographic exposure time of lanes A–C of Figure 4 was reduced from 72 to 24 h to yield lanes G–I, respectively, of Figure 4. Again no primer RNA was observed in the matrix sample of the RNA density region (Figure 4, lane H) even though the radiographic intensity of this lane was similar to that of the matrix sample of the DNA density region (Figure 3, lane B).

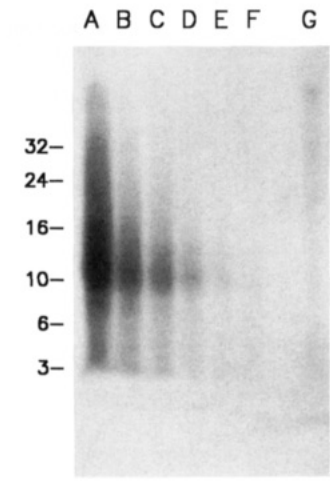

FIGURE 5: Autoradiogram of the relative amounts of total primer RNA in the matrix and nonmatrix fractions of the nucleus. Primer RNA was isolated from the matrix and nonmatrix fractions of the nucleus following DNase I digestion of the RNA-primed nascent DNA as described under Experimental Procedures. Lanes A–F: Serial dilutions corresponding to 20, 10, 5, 2.5, 1.25, and 0.63%, respectively, of the total primer RNA isolated from the nuclear matrices of 4×10^7 cells. Lane G: 20% of the total nonmatrix fraction obtained from the above tumor cells.

Figures 3 and 4 indicate that, on the basis of RNA content, the primer RNA was considerably enriched in the nuclear matrix compared to the nonmatrix fraction. In order to obtain a quantitative estimate of the relative amounts of total primer RNA in the matrix and nonmatrix fractions, serial dilutions of the nuclear matrix primer RNA were analyzed by electrophoresis. Lanes A and G of Figure 5 represent 20% of the total amount of the matrix and nonmatrix fractions isolated from 4×10^7 CCRF-CEM cells, respectively. A large band of primer RNA was seen in the matrix sample (lane A), whereas no definite band of primer RNA was visible in the nonmatrix sample (lane G). It was possible to dilute the matrix sample at least 16-fold and still detect primer RNA (lane E). Thus, the amount of primer RNA present in the nuclear matrix was at least 16-fold greater than in the high-salt-soluble nonmatrix fraction. Taken together, the results depicted in Figures 2–5 indicate that (a) greater than 94% of the primer RNA is present in the matrix fraction of the nucleus and (b) this primer RNA is functional since essentially all of it was extended into Okazaki fragments that banded in the DNA density region of the cesium chloride gradient.

Nuclear Matrix Primer RNA Is Covalently Linked to Nascent DNA. In order to specifically demonstrate the presence of a covalent linkage between primer RNA and nascent DNA is isolated from the nuclear matrix, a phosphate-transfer analysis was carried out. Newly replicated DNA was radiolabeled by incubating whole-cell lysates of logarithmically growing CCRF-CEM cells with [α-^{32}P]dTTP and [^3H]dTTP for 10 min. The nucleic acids were purified from the nuclear matrix and nonmatrix fractions, and aliquots from each fraction that contained equal amounts of total RNA

Table II: Distribution of ^{32}P in Ribonucleoside 2′(3′)-Monophosphates after Alkaline Hydrolysis of Nuclear Matrix DNA Labeled in Whole-Cell Lysates with [α-^{32}P]dTTP[a]

ribonucleoside 2′(3′)-monophosphate	^{32}P cpm transferred	% distribution[b]
2′(3′)CMP	552	25 ± 8
2′(3′)UMP	895	41 ± 5
2′(3′)GMP	490	23 ± 4
2′(3′)AMP	232	11 ± 1

[a] Whole-cell lysates prepared from exponentially growing CCRF-CEM cells were incubated for 10 min at 25 °C with [α-^{32}P]dTTP. Nuclear matrices were isolated and the nucleic acids were purified from the matrices by phenol/chloroform extraction and then heated in 0.05 M KOH for 45 min at 100 °C. The radiolabeled ribonucleoside 2′(3′)-monophosphate hydrolysis products were then separated by reversed-phase HPLC and quantitated by liquid scintillation counting. A total of 129 502 ^{32}P cpm was incorporated into DNA, of which a total of 2169 cpm (1.7%) was transferred to the four solubilized ribonucleoside 2′(3′)-monophosphates. The recovery of radioactivity from the HPLC column was 94%. [b] Results are the means of three experiments ± standard deviations.

were subjected to alkaline hydrolysis. If a covalent linkage existed between the primer RNA (on the 5′-side) and the nascent DNA, then the ^{32}P label that had been incorporated into the nascent DNA at the RNA/DNA junction would be transferred after alkaline hydrolysis to a solubilized ribonucleoside 2′(3′)-monophosphate. In contrast, no transfer of ^{3}H to a ribonucleoside monophosphate should occur, since this isotope was not present in the phosphodiester linkage. HPLC separation of the hydrolysis products from the nuclear matrix sample revealed that ^{32}P was transferred to each of the four ribonucleoside 2′(3′)-monophosphates (Table II). No transfer of ^{3}H to any of the ribonucleoside monophosphates was detected (data not shown). This was in agreement with earlier reports of a random distribution at the 3′-end of the primer RNA isolated from whole cells (Waqar & Huberman, 1975a,b; Tseng et al., 1979; Kitani et al., 1984). As one would expect, the amounts of ^{32}P transferred were low (1.7%), since only the ^{32}P label located at the RNA/DNA junction was transferred to a ribonucleoside 2′(3′)-monophosphate. A significant amount of phosphate transfer was not detected in the nonmatrix sample (data not shown), which was consistent with the absence of RNA-primed nascent DNA in this fraction of the nucleus (Figures 3–5).

Control Experiments. The results of this study provided evidence that primer RNA synthesis occurred on the nuclear matrix of CCRF-CEM cells. However, one might argue that the preferential association of primer RNA with the nuclear matrix was the result of aggregation of the primer RNA that was induced during high-salt extraction of the nuclei. The data obtained from several types of control experiments indicated that the nonspecific association of primer RNA with the nuclear matrix was unlikely. One would expect that if the primer RNA were merely salted-out along with the insoluble matrix proteins, then the repeated washing of the nuclear matrices with low-salt buffer should resolubilize the primer RNA. On the contrary, the primer RNA remained highly localized in the insoluble matrix fraction of the nucleus despite successive washings of the isolated matrices with high-salt buffer, low-salt buffer, and low-salt buffer that contained 1% Triton X-100 (Figures 3, 4, and 5).

Mixing experiments provided further evidence that neither newly replicated RNA nor RNA-primed nascent DNA aggregated with the nuclear matrix during the matrix isolation procedure. Total nuclear [^{32}P]RNA and [^{32}P]RNA-primed nascent DNA were isolated from nuclei and nuclear matrices, respectively. These RNAs, which had different degrees of proteinization (total nuclear RNA > RNA-primed nascent DNA), were added to unlabeled nuclei, and the nuclear matrices were then prepared. Table III indicates that only 0.1% of the total exogenous [^{32}P]RNA was recovered in the nuclear matrix fraction, whereas 62% of the total endogenous [^{32}P]-RNA was present in the nuclear matrix. Likewise, 7% of the added [^{32}P]RNA-primed nascent DNA was nonspecifically associated with the nuclear matrix, but about 98% of the endogenous [^{32}P]RNA-primed nascent DNA was present in the nuclear matrix sample following density gradient purification of the nuclear matrix and nonmatrix fractions. Thus, only a small fraction of the newly replicated RNA and RNA-primed nascent DNA that is recovered on the nuclear matrix could be the result of aggregation of these RNAs with the nuclear matrix. Taken together, the results presented in Figures 2, 3, and 5 and Tables I and III support the concept that primer RNA and RNA-primed nascent DNA are synthesized at DNA replication sites on the nuclear matrix.

Table III: Binding of Exogenous Newly Replicated RNA and RNA-Primed Nascent DNA to the Nuclear Matrix

nuclear RNA isolated	cellular fraction	mean cpm ($\times 10^{-2}$)	% of total cpm in nuclear matrix fraction[b]
[^{32}P]RNA (endogenous)	matrix	10532	62 (52–67)
	nonmatrix	6471	
[^{32}P]RNA (exogenous)	matrix	6	0.1 (0.06–0.12)
	nonmatrix	6173	
[^{32}P]RNA-primed DNA (endogenous)	matrix	1172	98 (96–100)
	nonmatrix	24	
[^{32}P]RNA-primed DNA (exogenous)	matrix	3	7 (4–8)
	nonmatrix	43	

[a] Whole-cell lysates were prepared from exponentially growing CCRF-CEM cells and pulse-labeled with [α-^{32}P]ATP. Nuclei were purified and the newly replicated [^{32}P]RNA and [^{32}P]RNA-primed nascent DNA were isolated as described under Experimental Procedures. The exogenous [^{32}P]RNA and [^{32}P]RNA-primed nascent DNA were mixed with aliquots of unlabeled nuclei, and the matrix and nonmatrix fractions were isolated from the unlabeled nuclei. Nonspecific association of the exogenous [^{32}P]RNA and [^{32}P]RNA-primed nascent DNA with the nuclear matrix and nonmatrix fractions was quantitated by liquid scintillation counting. The results obtained were compared to the amounts of endogenous [^{32}P]RNA and [^{32}P]RNA-primed nascent DNA measured in the matrix and nonmatrix fractions of the nucleus. [b] Mean of triplicate determinations with the range of values in parentheses.

It was also important to address the possibility that the lack of primer RNA in the nonmatrix fraction of the nucleus was the result of degradation of the nonmatrix primer by RNases. Data obtained from the mixing experiment described above indicated that no significant degradation of the primer RNA occurred. Aliquots of purified [^{32}P]RNA-primed nascent DNA (average of 617 900 cpm) were added in triplicate to unlabeled nuclei according to the standard procedure. The total recovery of [^{32}P]RNA-primed nascent DNA following ethanol precipitation of the matrix and nonmatrix fraction averaged 94% ± 1.8 SD (580 826 cpm). Reanalysis of the [^{32}P]RNA-primed DNA present in each of these fractions by gel electrophoresis revealed that the primer RNA was of normal length (8–10 nucleotides). Thus, no evidence was obtained for degradation and subsequent loss of the nonmatrix primer RNA during the isolation procedure.

Discussion

Various models of eukaryotic DNA replication have been proposed. One of the more interesting and plausible models is the nuclear matrix bound replication system. According to this model, all DNA replication takes place on the insoluble

nuclear matrix framework, and the newly replicated DNA moves progressively away from the matrix-bound sites at the rate of replication fork movement (Pardoll et al., 1980; Vogelstein et al., 1980; Berezney, 1984; Tubo et al., 1985; Razin, 1987). Since RNA-primed nascent DNA is associated exclusively with DNA replication forks, it was of interest to characterize the nuclear synthesis and distribution of RNA-primed nascent DNA as a means of specifically evaluating the importance of the nuclear matrix in DNA replication. We found a striking localization of both primer RNA and RNA-primed nascent DNA on the nuclear matrix, and none of these intermediates were detected in the high-salt-soluble fraction of the nucleus. At least 94% of the RNA-primed nascent DNA and 16% of the total nuclear RNA (as determined by reaction with orcinol) were associated with the nuclear matrix. On the other hand, at most 6% of the RNA-primed nascent DNA, but 84% of the total RNA, was present in the nonmatrix fraction of the nucleus. Thus, RNA-primed nascent DNA was enriched at least 80-fold in the nuclear matrix relative to the nonmatrix fraction of the nucleus when the results were normalized to the amount of RNA [(94/16)/(6/84) = 80]. Primer RNA content was observed to be at least 16-fold elevated in the nuclear matrix when the results were expressed as the relative amounts of total primer RNA present in the matrix and nonmatrix fractions. We also observed that 22% of the total nuclear DNA primase activity was tightly bound to the nuclear matrix following multiple washings of the isolated matrices with high- and low-salt buffers. Since the logarithmically growing CCRF-CEM cultures used in these studies contained 17% S-phase cells,[2] our finding that 22% of the nuclear primase activity was bound to the nuclear matrix seemed reasonable. The above observations, together with the almost exclusive localization of primer RNA on the nuclear matrix, are consistent with the nuclear matrix model of DNA replication.

Our finding of essentially no soluble (i.e., non-matrix-bound) RNA-primed nascent DNA could not be attributable to loss of these fragments during the isolation procedure. The recovery of either newly replicated DNA or exogenous RNA-primed nascent DNA was checked in all cellular fractions and purification steps, including nuclear isolation, phenol/chloroform extraction of the nuclear matrix and nonmatrix fractions, and density gradient centrifugation. No selective loss of newly replicated DNA from the nonmatrix fraction or degradation of exogenous RNA-primed nascent DNA added to the nonmatrix fraction was detected.

The length and base composition of primer RNA is thought to be regulated by the intracellular concentrations of the deoxyribonucleoside triphosphates (Yamaguchi et al., 1985; Hirose et al., 1988). The predominant primer RNAs that are recovered from eukaryotic cells are of fairly discrete length of 8–10 nucleotides (Tseng & Goulian, 1977; Tseng et al., 1979; Kitani et al., 1984). In this paper we show that the nuclear matrix is highly enriched in primer RNA following incubation of whole-cell lysates containing intact nuclei with the four ribo- and deoxyribonucleoside triphosphates. Analysis of purified preparations of nuclear matrix primer RNA showed that this primer RNA was of normal length (8–10 nucleotides) and functional, since essentially all of the primer RNA was extended with DNA into Okazaki fragments. These results differ somewhat from that observed following incubation of isolated nuclear matrices with only one of the ribonucleoside triphosphates (Tubo & Berezney, 1987c). Under these latter conditions, mixtures of oligoribonucleotide products of 8–50 nucleotides are formed.

The nonmatrix or high-salt-soluble fraction of the nucleus contained an insignificant amount of RNA-primed nascent DNA but most of the newly replicated DNA. These results suggested that the primer RNA is degraded prior to the migration of the nascent DNA away from the matrix-bound DNA replication sites. It has been reported that 27% of the RNase H activity was specifically associated with the nuclear matrix of regenerating rat liver (Tubo & Berezney, 1987a). RNase H activity is thought to catalyze the removal of the primer RNA prior to the ligation of the Okazaki fragments into higher molecular weight DNA (DiFrancesco & Lehman, 1985). Therefore, we propose that both RNA-primed nascent DNA synthesis and the removal of the primer RNA from the Okazaki fragments are nuclear matrix functions. The studies described herein do not directly address the possibility of subsequent ligation of the Okazaki fragments on the nuclear matrix. However, DNA ligase activity was not detected in the matrix fraction of nuclei from either normal or regenerating rat liver (Tubo & Berezney, 1987a).

This laboratory, as well as several others, has reported that the nuclear matrix of proliferating cells is enriched in newly replicated DNA relative to either the whole nucleus or the high-salt-soluble fraction of the nucleus (Berezney & Coffey, 1974, 1975; Pardoll et al. 1980; Foster & Collins, 1985; Fernandes et al., 1988). This evidence is also consistent with the nuclear matrix model of DNA replication. Alternative explanations for the apparent enrichment of newly replicated DNA with the nuclear matrix that are not consistent with this model have been proposed. One of these concerns the possible nonspecific aggregation of the primer RNA and RNA-primed nascent DNA with the nuclear matrix that is promoted by the high-salt matrix isolation procedure (Djondjurov et al., 1986). Our finding of an almost totally selective association of primer RNA and RNA-primed nascent DNA with the nuclear matrix would argue against the nonspecific binding of these intermediates to the matrix. The nonmatrix fraction of the nucleus contained most of the nascent DNA but an insignificant amount of either primer RNA or RNA-primed nascent DNA. Thus, it would seem highly unlikely that nearly all of the RNA-primed nascent DNA but only a minor fraction of the nascent DNA would aggregate with the nuclear matrix. This study and previous work from this laboratory (Fernandes et al., 1988) have also demonstrated that neither exogenous nuclear RNA, RNA-primed nascent DNA, chromatin fragments, nor nuclear matrix DNA bound significantly to the nuclear matrix of CCRF-CEM cells. Instead, these observations are more consistent with the synthesis of RNA-primed nascent DNA on the nuclear matrix and the removal of the primer RNA before the nascent DNA moves into the bulk DNA. One might also argue that the RNA-primed nascent DNA, but not the nascent DNA, is associated with the replicative DNA polymerase α–DNA primase megacomplexes (Tubo & Berezney, 1987b) and these complexes are precipitated along with the nuclear matrix during the high-salt extraction procedure. On the contrary, Collins and Chu observed that neither exogenous DNA polymerase α nor DNA primase precipitated with the nuclear matrix fraction following the high-salt extraction and DNase I digestion of nuclei (Collins & Chu, 1988).

Detailed studies of primer RNA synthesis in intact cells or nuclei have previously been difficult to carry out because of the very low concentrations of the primer within cells and the relatively large amounts of other RNAs. Our finding of a high

[2] D. J. Fernandes, unpublished observations.

degree of enrichment of primer RNA on the nuclear matrix should facilitate further investigations into the mechanisms of discontinuous DNA synthesis in eukaryotic cells. In addition, the presence of DNA primase in matrix-associated megacomplexes (Tubo & Berezney, 1987b) will provide the opportunity for examining the functional interactions of DNA primase with the other enzymes involved in DNA replication.

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Registry No. DNA primase, 64885-96-7.

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