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Biochemistry

Initiation of HeLa cell DNA synthesis in a subnuclear system

(DNA replication/reconstructed nuclei/initiator RNA/RNA polymerase I)

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ABSTRACT **Mammalian cells are known to synthesize DNA in discrete stages, the first of which seems to be the formation of DNA pieces 150–200 nucleotides in length that have a s_{20} value of about 4 S. We have reconstructed a system derived from HeLa cell nuclei that carries out RNA-primed initiation of the synthesis of small (4S) DNA fragments. This synthesis is resistant to high concentrations of α-amanitin and sensitive to antibody directed against RNA polymerase I, suggesting that this enzyme may be involved in the initiation step. The formation of small DNA fragments in this system also requires DNA polymerase α, heat-labile nuclear factor(s), and at least one other nuclear protein.**

Systems used to study the mechanisms of DNA replication of eukaryotic cells include permeabilized cells (1, 2), cell lysates (3–6), and isolated nuclei (7–10). Despite disruption of the cell structure, isolated nuclei support semiconservative DNA synthesis (11, 12), the discontinuous process of viral and/or cellular DNA replication (13–15), and the initiation of viral Okazaki pieces by RNA primers (16, 17) and their conversion into longer DNA chains (14, 18, 19). Moreover, Seki and Mueller (20), have reported that the "replicase" activity of nuclei can be partly solubilized by mild salt treatment.

In this report we describe optimal conditions for solubilization of the DNA replicase activity and for reconstruction of the DNA synthetic activity of isolated nuclei from HeLa cells. Using such a reconstructed nuclear system, we have found conditions in which DNA synthesis is dependent upon the addition of rNTPs. We present evidence for the involvement of rNTPs in the priming event for DNA synthesis and can now identify at least four components in the initiation step of mammalian DNA synthesis. These components include DNA polymerase α, a RNA polymerase activity with the properties of RNA polymerase I, a heat-labile factor(s) tightly bound to nuclei, and a fourth component solubilized by salt extraction of nuclei.

MATERIALS AND METHODS

Nuclei Preparation. HeLa S_3 cells were synchronized by the double thymidine block previously described (21) and collected in mid S phase 5 hr after release of the second thymidine block. Nuclei were prepared from these cells according to the procedure described by Krokan *et al.* (8), except that Hepes buffer, pH 7.5, was used in place of Tris·HCl, pH 7.5. The detergent-treated nuclei were thoroughly washed in isotonic buffer, pelleted, and stored in 200×10^6 nuclei aliquots in liquid nitrogen.

Nuclear Salt Treatment. Nuclei (200×10^6 in 0.5 ml) were suspended in 3 vol of buffer A [10 mM Hepes, pH 7.5/3 mM $MgCl_2$/2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)/1 mM dithioerythritol] containing 0.32 M NaCl and gently stirred for 10 min. After centrifugation at $1000 \times g$ for 10 min, they were resuspended in 5–8 vol of buffer A containing 10% (vol/vol) glycerol and 0.27 M NaCl and stirred for an additional hr at 0–4°. This operation was repeated once with a 30-min stirring period. The nuclei were then washed in 10 vol of buffer A with 10% glycerol and 50 mM NaCl and finally suspended in this mixture at $60–150 \times 10^6$ nuclei per ml.

The supernatant obtained after the first salt treatment of nuclei with 3 vol of buffer A containing 0.32 M NaCl was cleared by centrifugation at $150,000 \times g$ for 30 min and desalted by passage through Sephadex G-25. The excluded fraction constitutes the nuclear salt extract; it contained the replicase activity used in the experiments in this paper. Alternatively, the nuclear salt extract can be precipitated with ammonium sulfate (30–55% saturation) and used after desalting with Sephadex G-25 as described above.

RESULTS

Properties of the Reconstructed Nuclear Replication System. Nuclei isolated from HeLa cells synchronized in S phase have been shown to continue, *in vitro*, the DNA replication process at the sites that were active *in vivo* (7). When these nuclei were extracted with 0.27 M NaCl as described in *Materials and Methods*, they retained less than 10% of their original rate of DNA synthesis. Alone, neither the nuclear salt extract obtained in the first salt treatment nor the salt-treated nuclei themselves (Fig. 1) were capable of DNA synthesis. When the salt-treated nuclei and the nuclear salt extract were recombined, substantial recovery of activity was observed (Fig. 1). Parallel experiments with nuclei from cells arrested in G_1 show them to have 20% of the activity of S-phase nuclei in the reconstructed system. By using a concentrated nuclear salt extract, the initial activity of the nuclei could be reconstituted when 3–5 nuclear equivalents of the nuclear extract were added back to the salt-treated nuclei from S-phase cells. The requirements and optimal conditions for DNA synthesis in the reconstructed nuclear system were found to be identical to those for DNA replication in isolated nuclei (legend to Fig. 1). When purified HeLa cell DNA polymerase α, β, or γ or RNA polymerase I, II, or III was added to the salt-treated nuclei in place of the nuclear salt extract, no increase in the DNA synthetic activity was observed. These results suggest that the nuclear salt extract contained other factors in addition to the DNA and RNA polymerase activities that were required for maximal activity of the DNA replication complex. Direct enzymatic analysis of the salt-treated nuclei and the nuclear salt extract showed that 64% of RNA polymerase I, 93% of II, and 22% of III remained in the salt-extracted nuclei, whereas 1%, 3%, and 15% of DNA polymerases α, β, and γ were still present. The rest of these polymerase activities were found in the nuclear salt extract. Because the necessary components in the nuclear salt extract are nondialyzable, precipitable by ammonium sulfate, and trypsin sensitive, we assume they are other protein factors.

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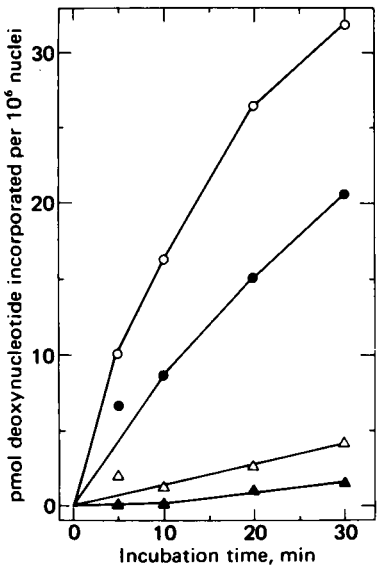

FIG. 1. Kinetics of DNA synthesis in the reconstructed nuclear system. The salt-treated nuclei (1.5×10^6 per 0.3-ml incubation mixture) were incubated in 50 mM Tris·HCl, pH 8.0/8 mM $MgCl_2$/6.5 mM ATP/2 mM dithiothreitol/50 mM NaCl/100 μM (each) of the four dNTPs containing [^{3}H]dTTP (567 cpm/pmol) and 45 μl of the nuclear salt extract (3.7 mg of protein per ml). At the end of incubation, 50-μl samples of the reaction mixtures were spotted on Whatman GFC filters, immersed in 5% trichloroacetic acid/1% sodium pyrophosphate solution, and further washed as described (22). The radioactivities on the filters were measured in a toluene-based scintillation medium. The results have been normalized for 10^6 nuclei. △, Salt-treated nuclei alone; ▲, 3 nuclear salt extract equivalents (i.e., derived from 4.5×10^6 nuclei) alone; ●, salt-treated nuclei with 3 equivalents of the nuclear salt extract; ○, control incorporation with 1.5×10^6 nuclei.

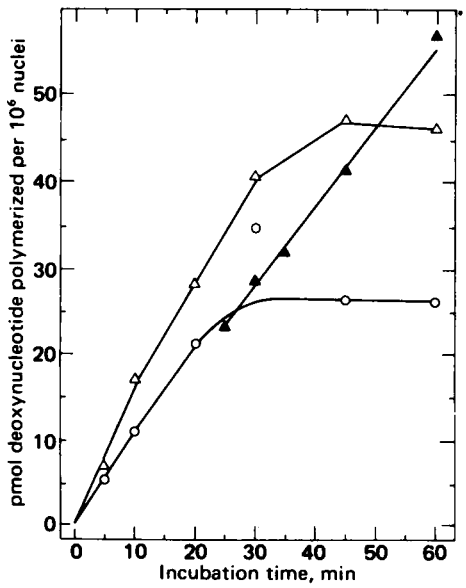

FIG. 2. Effect of rNTPs on DNA synthesis in the reconstructed system. Reaction mixtures containing 8×10^6 salt-treated nuclei in 0.4 ml with 2.5 nuclear salt extract equivalents were incubated under standard assay conditions as described in the legend to Fig. 1. A mixture of [^{3}H]dTTP (660 cpm/pmol) and the four rNTPs (0.3 mM each) was added at the beginning of the incubation (△) or after 25 min of incubation (▲). ○, Kinetics of DNA synthesis under the same conditions but without addition of rNTPs. Aliquots (50 μl) of the incubation mixture were acid precipitated and assayed for radioactivity at the indicated times.

Furthermore, we have also found that incubation of the extracted nuclei at 45°C for 2 min completely abolished their ability to participate in the reconstituted replication system. These conditions do not affect the DNA or RNA polymerase activities contained in the salt-extracted nuclei and show the need for an additional heat-labile factor that is not removed by salt extraction of the nuclei.

Effect of rNTP Addition in the Reconstructed Nuclear System. Using the reconstructed nuclear system, we found that addition of rNTPs at the beginning of the incubation enhanced the rate of DNA synthesis by 20–30%. This increase in rate of DNA synthesis was followed by an increase in the yield of DNA synthesized (Fig. 2). However, we have been able to dissociate almost completely the *de novo* initiation of Okazaki pieces from the elongation of preformed DNA chains in the reconstructed system. This is presented in Fig. 2, where it can be seen that DNA synthesis in the reconstituted system almost ceased after about 30 min, presumably due to the end of the elongation of preformed DNA fragments. If rNTPs were then added at 30 min, DNA synthesis resumed immediately and continued for an additional 20–30 min. The stimulatory effect of the rNTPs was dependent upon their concentration. The high concentration of ATP (6.5 mM) already present in the DNA-synthesizing system made further addition of ATP unnecessary. Complete omission of ATP reduced DNA synthesis to 10% of that at maximum. Optimal concentration was found to be 0.5 mM of each of the other three added rNTPs. Omission of one rNTP lowered the stimulation to about 60% and omission of two of the rNTPs to about 20% of the control value. Increasing the concentration of the corresponding dNTP when one or two rNTPs were omitted did not result in a significant effect, showing that the stimulatory effect observed was specific for the rNTPs.

Analysis of the DNA Products Synthesized in the Presence of rNTP. We have analyzed the DNA products synthesized in the reconstructed nuclear system after a preincubation for 45 min. The reaction was first carried out for 45 min at 37°C with the unlabeled dNTP precursors for DNA synthesis. The nuclei were then pelleted and resuspended in complete incubation mixture with fresh nuclear salt extract, with or without rNTP. Newly synthesized DNA was pulse-labeled for 2 min with [^{3}H]dTTP, and the pulse-labeled DNA products were analyzed on alkaline sucrose velocity gradients. Fig. 3*A* shows the pattern of sedimentation of DNA pulse-labeled in the absence of rNTPs. The small amount of DNA formed sedimented as a broad peak in the 3–18S region and probably reflects residual elongation of preformed DNA or repair synthesis in gapped regions of the parental DNA. When the same reaction is carried out in the presence of added rNTPs, a 2- to 3-fold stimulation of DNA synthesis was observed. This stimulatory effect was found to be due to the synthesis of small DNA fragments sedimenting at 3–5 S with an average value of 4.2 S, corresponding to 170 nucleotides in length (Fig. 3*B*). Previous experiments with isolated nuclei have shown that cytoplasmic proteins catalyze elongation of DNA chains (14, 18, 19). A chase experiment, in which added HeLa cytoplasm protein and a 20-fold excess of unlabeled dTTP were added for 10 min, showed that significant numbers of these short pieces of DNA were elongated to an average size of 10.5 S (about 1700 nucleotides).

To obtain more information about the enzymatic steps, different inhibitors of RNA polymerase activity were used, and their effects on the formation of Okazaki pieces in the presence of rNTPs were analyzed. As shown in Fig. 3*C*, under conditions in which RNA polymerase II and III were completely inhibited by high levels of α-amanitin (0.1 mg/ml), the rNTP-dependent formation of the 4S DNA pieces still occurred; in fact, it was slightly stimulated. Two simple explanations can be advanced for the synthesis of RNA in the presence of α-amanitin. Either

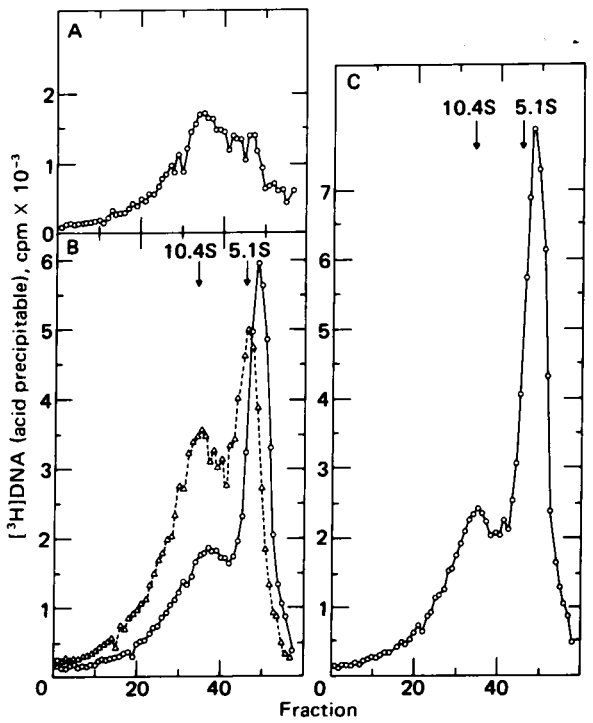

FIG. 3. Alkaline sucrose gradient analysis of the DNA product synthesized in the presence or absence of rNTP. Salt-treated nuclei (8×10^6 per 200-μl incubation mixture) were preincubated for 45 min at 37°C with 3 nuclear salt extract equivalents in the presence of unlabeled dNTP [100 μM each, except dTTP (50 μM)] under standard assay conditions (Fig. 1). After the preincubation, the nuclei were pelleted at 1000 $\times g$ for 10 min and resuspended in the complete incubation mixture (+ or − rNTPs) with 2 nuclear salt extract equivalents but without dTTP. After incubation for 5 min at 37°C, the DNA was labeled for 2 min with 20 μM [^{3}H]dTTP containing 100 μCi (1 Ci = 3.7×10^{10} becquerels). The reaction was stopped by addition of 1 vol of 40 mM EDTA/0.5 mg of proteinase K per ml/0.2% Sarkosyl and then diluted 1:2 with a buffer containing 20 mM EDTA, 0.25 mg of proteinase K per ml, and 0.1% Sarkosyl. After 2 hr at 55°C, the incubation mixture was made 0.2 M in NaOH, heated at 55°C for 5 min, and layered onto a 13-ml 5–20% sucrose gradient containing 0.9 M NaCl, 1 mM EDTA, 0.1% Sarkosyl, and 0.2 M NaOH. The sedimentation was carried out for 6 hr at 38,000 rpm in a Beckman SW 40 rotor. Fifty-six fractions of 250 μl each were collected from the bottom of the tubes and 100 μl of each fraction was precipitated and assayed for radioactivity. The recovery was more than 90%. Sedimentation marker DNA (vaccinia [^{14}C]DNA) fragments were run in parallel gradients. (*A*) No rNTPs added. (*B*) With 0.5 mM UTP, GTP, and CTP. ○, Two-minute pulse label; △, 2-min pulse label followed by 10-min chase with unlabeled dTTP in the presence of 240 μg of cytoplasm protein (7). (*C*) As 2-min pulse label in *B* plus 0.1 mg of α-amanitin per ml.

RNA polymerase I [or an unknown "primase" (23) that is insensitive to α-amanitin] catalyzes the synthesis of a primer RNA or RNA polymerase II, RNA polymerase III, or both are capable of synthesizing the small RNA primers in the presence of α-amanitin. We feel the latter possibility to be unlikely because we have been unable to detect synthesis of any small (or large) RNA fragments by RNA polymerase II or III with purified enzymes in the presence of α-amanitin. To further investigate the first possibility, we have preincubated both salt-treated nuclei and the nuclear salt extract with antibody directed against RNA polymerase I and then examined the DNA synthesized under conditions optimal for the rNTP stimulation of DNA synthesis. Fig. 4 shows that a significant inhibition (40–60%) of the rNTP-dependent DNA synthesis occurs when antiserum to RNA polymerase I is used as compared to a preimmune control serum, which has no effect on the synthesis

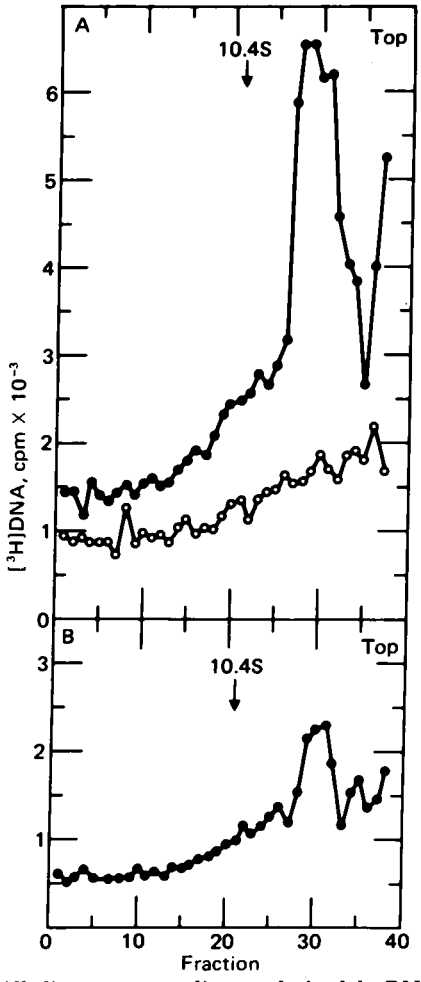

FIG. 4. Alkaline sucrose gradient analysis of the DNA products synthesized in the presence of anti-RNA polymerase I immunoglobulins. Salt-treated nuclei (6×10^6/200 μl) were preincubated with 375 μg of rabbit control (preimmune) immunoglobulin (*A*) or 375 μg of rabbit anti-calf thymus RNA polymerase I immunoglobulin (*B*). This latter immunoglobulin has been shown to crossreact with HeLa cell RNA polymerase I (24). The nuclei were then pelleted and resuspended in an incubation mixture under conditions described in the legend to Fig. 3. Three nuclear salt extract equivalents that had been preincubated for 30 min at 0°C with 400 μg of control immunoglobulin or 400 μg of calf thymus anti-RNA polymerase I immunoglobulin were used. After 10 min at 37°C, [^{3}H]dTTP (75 μCi, 41 μCi/nmol) was added and the DNA was pulse labeled for 2 min. The reaction mixture was set up and processed as described in the legend to Fig. 3 and an aliquot was centrifuged in a 5–20% alkaline sucrose gradient for 90 min at 45,000 rpm in Beckman VTi 65 rotor. Results are expressed as total acid-insoluble [^{3}H]DNA cpm per fraction. (*A*) ○, Control reaction without immunoglobulins and rNTP; ●, reaction carried out in the presence of preimmune immunoglobulins and 0.5 mM rNTP. (*B*) ●, DNA synthesized in the presence of anti-RNA polymerase I immunoglobulins and 0.5 mM rNTP.

of DNA in the reconstructed system. Furthermore, it is the synthesis of the 4S DNA that is markedly reduced by the antiserum to RNA polymerase I (Fig. 4*B*). Though not definitive, these experiments suggest that RNA polymerase I must be considered as a possible "primase" for mammalian DNA synthesis.

Additional experiments with the arabinose analogs of ATP and CTP, which are known to specifically inhibit DNA polymerase α (25) under proper conditions, show that they also stop DNA synthesis in the reconstructed nuclear system at concentrations of 200 μM (data not shown). Other groups (26, 27) also have noted that DNA polymerase α is involved in the chain elongation of simian virus 40 DNA and HeLa DNA.

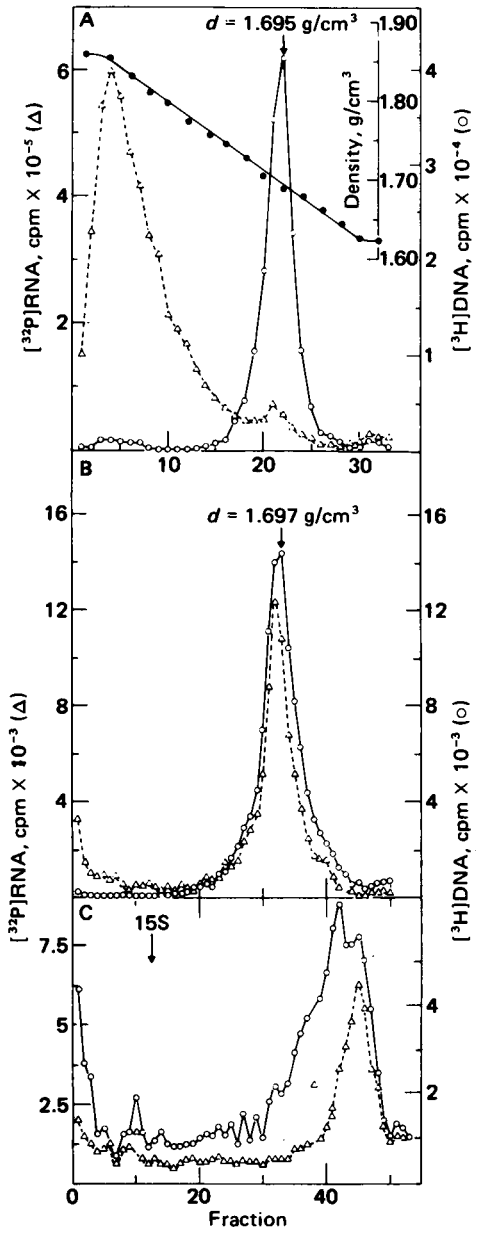

FIG. 5. Purification and sizing of the [^{32}P]RNA-[^{3}H]DNA product synthesized with the reconstructed nuclei. The salt-treated nuclei (6×10^6 per 200-μl incubation mixture) were preincubated for 45 min at 37°C in the presence of the nuclear salt extract, the complete incubation mixture (Fig. 1), 100 μM dATP, dCTP, dGTP, and 50 μM dTTP. The nuclei were then pelleted and resuspended in a 200-μl incubation mixture containing 0.1 mg of α-amanitin per ml; all four rNTPs with 600 μM ATP and GTP, 65 μM [α-^{32}P]CTP and [α-^{32}P]UTP (both 79 μCi/nmol); and 100 μM each dATP, dCTP, and dGTP. The suspension was then incubated for an additional 5 min at 37°C. After this time, [^{3}H]dTTP (35 μM, 23 μCi/nmol) was added and DNA was labeled for 2 min. The reaction mixture was processed with proteinase K, phenol treated, and ethanol precipitated. After ethanol precipitation, the deproteinized [^{32}P]RNA-[^{3}H]DNA product was suspended in 50 mM potassium phosphate, pH 7.4/5 mM EDTA, heat denatured for 5 min at 100°C, and chilled immediately in ice. Then HCHO was added to a final concentration of 0.3 M.

Isopycnic centrifugation. (*A*) Labeled DNA product was centrifuged for 45 hr at 40,000 rpm in a Spinco type 70 rotor in a mixture made up of 5 ml of the [^{32}P]RNA-[^{3}H]DNA product, 17.5 ml of saturated CsCl solution, and 2.5 ml of saturated Cs_2SO_4 solution in 50 mM potassium phosphate, pH 7.4/5 mM EDTA/0.3 M HCHO. Δ, [^{32}P]RNA; ○, [^{3}H]DNA. Values in the ordinate represent the total acid-precipitable radioactivity per fraction. (*B*) The peak fractions 18–25 from gradient *A* were pooled, heat denatured at 55°C, and rebanded under the same conditions as for *A*. Recovery was 80% for [^{3}H]DNA and 35% for [^{32}P]RNA. Δ, [^{32}P]RNA; ○ [^{3}H]DNA. Values in the ordinate represent the total acid-precipitable radioactivity per fraction.

Sucrose gradient sedimentation. (*C*) Pooled peak fractions from gradient *B* (fractions 26–38) were dialyzed against 50 mM potassium phosphate/5 mM EDTA, pH 7.0, after addition of 0.25 mg of native calf thymus DNA per ml. They were then made 0.5 M in glyoxal and heat treated at 60°C for 20 min. This preparation was centrifuged in a 5–20% sucrose gradient containing 0.1 M glyoxal, 20 mM potassium phosphate at pH 7.4, and 0.1% Sarkosyl, for 14 hr at 30,000 rpm at 20°C in a Beckman SW 40 rotor. Fractions were collected from the bottom of the tube. ○, [^{3}H]DNA; Δ, [^{32}P]RNA. Values reported represent the total radioactivity per gradient fraction.

Evidence that rNTPs Are Involved in RNA Priming of Okazaki Pieces. Two approaches have been used to analyze the fate of added rNTPs and their relationship to formation of 4S DNA. The first one involved the use of [α-^{32}P]rNTPs and [^{3}H]dTTP to label both RNA and DNA after a 45-min preincubation of the reconstructed system. The reaction was carried out in the presence of α-amanitin and the nucleic acids were pulse labeled for 2.5 min. The pulse-labeled covalently linked RNA-DNA products synthesized in the reconstructed system were isolated, subjected to heat denaturation, and then centrifuged under denaturing conditions in a CsCl/Cs_2SO_4 density gradient. The labeled DNA peak fractions were pooled and rebanded under the same conditions. Fig. 5 *A* and *B* shows the pattern of banding of the [^{32}P]RNA-[^{3}H]DNA product. After the second centrifugation, 0.1–0.5% of the acid-insoluble [^{32}P]RNA formed in the presence of α-amanitin still copurified with the DNA product. The labeled material recovered from the second rebanding in Cs_2SO_4 was denatured in the presence of glyoxal and centrifuged through a sucrose/glyoxal gradient under conditions in which RNA appears to be totally denatured (28). The pattern of centrifugation presented in Fig. 5*C* shows that most of the [^{32}P]RNA, which was found to be alkali sensitive, still remained associated with the short DNA fragments. Unexpectedly, the [^{32}P]RNA peak did not coincide with the size distribution of the DNA pieces. [^{32}P]RNA was found to be associated with the shortest fragments, whose average S value was 3.2 S. The molar ratio of DNA to RNA in these fractions was 17.5. The simplest explanation of these results is that RNA is covalently linked to the short DNA fragments. In support of this, we have found, using gel electrophoresis (4), that DNase digestion of the 3–4S [^{32}P]RNA-[^{3}H]DNA, obtained as shown in Fig. 5*C*, shifts the ^{32}P-labeled material from an average size of 70–150 nucleotides to a size of 8-15 nucleotides.

The second approach used to determine the nature of the RNA–DNA association was to perform a ^{32}P transfer experiment from DNA to RNA to demonstrate a covalent linkage between the two molecules. The DNA was pulse labeled with each of the four [α-^{32}P]dNTPs separately and the DNA products were isolated after the unreacted dNTP had been removed by Sephadex filtration. Following alkali digestion, the products were subjected to paper electrophoresis to isolate ribonucleoside 2′- or 3′-monophosphates. The resulting ^{32}P-labeled products migrating at the positions of either 2′ or 3′ CMP, AMP, GMP, or UMP were identified by autoradiography and their radioactivities were measured. We were able to find α-^{32}P transfer from DNA to RNA with all four [α-^{32}P]dNTPs used (data not shown). The 16 possible combinations expected for a random sequence distribution of RNA-DNA linkages were found, indicating absence of a site-specific initiation of the 4S DNA fragments. This is in agreement with other studies on RNA priming in eukaryotic cells (16, 17). From the percentage of ^{32}P transfer from DNA to RNA (0.36–0.75%), we could calculate that, on the average, one RNA-DNA link existed for

every 200-nucleotide-long DNA piece, which is in agreement with the DNA/RNA ratio of the purified 4S DNA pieces if the primer RNA is 8–15 nucleotides long.

DISCUSSION

Until now, *de novo* initiation of Okazaki fragments in isolated nuclei has been demonstrated only in the synthesis of polyoma DNA, in which short RNA primers (an average of 10 nucleotides long) were found associated with the 5′ end of the isolated 4S DNA fragments (17). The experiments reported in this paper provide evidence for a direct involvement of RNA in the priming of cellular Okazaki fragments. Our reconstructed system shows a substantial dependence on added rNTPs for initiation of DNA synthesis, because omission of one rNTP resulted in a 40% inhibition and omission of two in a 70–80% inhibition of new 4S DNA synthesis. The 4S molecules can be elongated in this system to give large DNA molecules and, therefore, behave like "Okazaki fragments" (4).

In our nuclear system, the stimulation of 4S DNA synthesis by rNTPs was completely insensitive to α-amanitin. Because α-amanitin is a potent inhibitor of RNA polymerases II and III, but not RNA polymerase I, we suggest that RNA polymerase I or an undetected "primase" (29) be considered as a candidate for the RNA priming process. We also have observed a significant inhibition of the formation of 4S DNA in the presence of antiserum directed against RNA polymerase I, which suggests that this enzyme participates in the synthesis of the primer RNA. Study of yeast RNA polymerase I has revealed the existence of an associated RNase H activity that could catalyze the removal of the primer RNAs during DNA replication (30). Moreover, it has recently been reported that yeast RNA polymerase I is highly efficient in promoting *in vitro* initiation of DNA synthesis of phage fd DNA as well as other DNAs (31).

The reconstructed nuclear system seems promising for characterization of the protein factors involved in eukaryotic DNA replication. Because the addition of the known purified HeLa cell DNA or RNA polymerases does not replace the nuclear salt extract in our reconstructed system, other proteins present in the nuclear salt extract must also be required for DNA synthesis. In addition, thermal denaturation studies indicate that one (or more) heat-labile proteins tightly bound to chromatin are required for DNA replication.

In summary, our reconstructed HeLa nuclear system, which initiates DNA synthesis, is, as expected, a multi-component complex that includes (*i*) DNA polymerase α, (*ii*) a "priming" RNA polymerase (perhaps RNA polymerase I), (*iii*) an additional protein factor(s) in the nuclear salt extract, and (*iv*) a heat-labile factor(s) and other factors that remain associated with the residual nuclear structure after salt extraction of the nuclei.

We are indebted to Drs. Claude Kedinger and Pierre Chambon for the antiserum to calf thymus RNA polymerase I.

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