Grimm, L., and Grassmann, W. (1964), *Hoppe-Seyler's Z. Physiol. Chem. 337,* 161.
Hannig, K., and Nordwig, A. (1967), in Treatise on Collagen, Vol. 1, Ramachandran, G. N., Ed., London, Academic Press, p 73.
Hulmes, D. J. S., Miller, A., Parry, D. A. D., Piez, K. A., and Woodhead-Galloway, J. (1973), *J. Mol. Biol. 79,* 137.
Inagami, T., and Murakami, K. (1972), *Anal. Biochem. 47,* 501.
Miller, E. J. (1971), *Biochemistry 10,* 1652.
Miller, E. J. (1972), *Biochemistry 11,* 4903.
Miller, E. J. (1976), *Mol. Cell. Biochem. 13,* 165.
Miller, E. J., and Lunde, L. G. (1973), *Biochemistry 12,* 3153.
Miller, E. J., and Piez, K. A. (1966), *Anal. Biochem. 16,* 320.
Piez, K. A. (1968), *Anal. Biochem. 26,* 305.
Pisano, J. J., Bronzert, T. J., and Brewer, H. G., Jr. (1972), *Anal. Biochem. 45,* 43.
Prockop, D. J., Berg, R. A., Kivirikko, K. I., and Uitto, J. (1976), in Biochemistry of Collagen, Ramachandran, G. N., and Reddi, A. H., Ed., New York, N.Y., Plenum Press, p 163.
Schroeder, W. A. (1967), *Methods Enzymol. 11,* 351.
Traub, W. (1974), *Isr. J. Chem. 12,* 435.
Traub, W., and Fietzek, P. P. (1976), *FEBS Lett. 68,* 245.
Trelstad, R. L., Kang, A. H., Igarashi, S., and Gross, J. (1970), *Biochemistry 9,* 4993.

RNA Primers in SV40 DNA Replication: Identification of Transient RNA–DNA Covalent Linkages in Replicating DNA†

Stephen Anderson, Gabriel Kaufmann,‡ and Melvin L. DePamphilis*

ABSTRACT: SV40 DNA, replicating in isolated nuclei, contains RNA–DNA covalent linkages which were quantitated by measuring the release of [2′(3′)-^{32}P]rNMPs from [^{32}P]-DNA incubated in KOH (^{32}P-label transfer assay). More than 96% of the ^{32}P label released during this incubation was shown to be in [2′(3′)-^{32}P]rNMPs by chemically converting it into cyclic [2′:3′-^{32}P]rNMPs and then enzymatically cleaving the cyclic nucleotides to produce [3′-^{32}P]rNMPs. [α-^{32}P]dNTP, incorporated into DNA, was identified as the ^{32}P donor because the amount of ^{32}P-label transferred was proportional to the specific radioactivity of the labeled substrate. All 16 possible rN–dN linkages were found in SV40 replicating DNA at frequencies that suggested a near-random distribution on the genome. These RNA–DNA covalent linkages behaved as transient intermediates in DNA synthesis; they disappeared at the same rate that nascent 4S DNA chains ("Okazaki pieces") were joined to the growing daughter strands. Therefore, these linkages exhibited kinetic properties consistent with the proposed role of RNA as a primer for discontinuous DNA synthesis. When 4S DNA joining was inhibited by the absence of cytosol, the disappearance of RNA–DNA covalent linkages was not prevented. Inhibition of DNA synthesis with either *ara*-CTP or *ara*-ATP also failed to block the removal of RNA–DNA covalent linkages. Thus, the excision of these putative RNA primers does not appear to require either the concomitant joining of 4S DNA chains or DNA synthesis.

DNA synthesis generally occurs contemporaneously on both sides of a replication fork during semiconservative DNA replication. This requires one of the daughter strands to grow in the 3′ to 5′ direction, despite the fact that all known DNA polymerases synthesize DNA only in the 5′ to 3′ direction. Okazaki and his co-workers (1968) reconciled these observations by postulating a mechanism of discontinuous DNA synthesis, whereby short pieces of nascent DNA are repeatedly initiated. This allows DNA synthesis to proceed simultaneously away from as well as toward the replication fork. However, none of the known DNA polymerases are able to initiate DNA synthesis de novo; synthesis always requires a 3′-OH terminated polynucleotide "primer" hydrogen-bonded to the template strand. The primer may be provided by the synthesis of an oligoribonucleotide for each nascent DNA chain. Such putative RNA primers must be transient since mature nonreplicating DNA does not contain RNA–DNA covalent linkages.

Recent work in eukaryotic DNA replication has been directed toward identifying and isolating oligoribonucleotides covalently linked to the 5′ termini of newly synthesized DNA chains (Pigiet et al., 1974; Hunter and Francke, 1974a; Reichard et al., 1974; Tseng and Goulian, 1975a; Waqar and Huberman, 1975a,b; Kaufmann et al., 1977; general review of RNA primers, Kornberg, 1976). The RNA priming hypothesis predicts that such putative RNA primers will be excised at a rate equal to or faster than the rate that short nascent pieces of DNA are joined to growing daughter strands. To test this notion, we have undertaken a study of the removal of RNA primers using nuclei isolated from SV40[1]-infected CV-1 cells.

† From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received June 23, 1977.* This work was supported by the National Institutes of Health Grant CA 15579-03. S. A. was supported by National Service Award CA 09031. G. K. was supported in part by a fellowship from the European Molecular Biology Organization. M. L. D. is an Established Investigator of the American Heart Association.

‡ Present address: Department of Biochemistry, The Weizmann Institute of Science, Rehovot, Israel.

[1] Abbreviations used: SV40, Simian virus 40; SV40(I) DNA, covalently closed superhelical viral DNA; SV40(II) DNA, duplex circular viral DNA containing at least one single-strand interruption; SV40(RI) DNA, replicative intermediates of SV40 DNA; Hepes-Na, sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate; EDTA, sodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; NTP and dNTP, ribo- and deoxyribonucleoside triphosphates, respectively; *ara*-CTP, cytosine 1-β-D-arabinoside-5′-triphosphate; *ara*-ATP, adenine 9-β-D-arabinoside 5′-triphosphate.

In this system, SV40 DNA replicating intermediates are faithfully converted into covalently closed, superhelical, viral DNA molecules when incubated with the four dNTPs, ATP, an ATP regenerating system, and a buffered salts solution (DePamphilis et al., 1975). Production of mature viral DNA requires a cytosol fraction, presumably containing factors released from the nucleus (DePamphilis and Berg, 1975). Addition of rCTP, rGTP and rUTP stimulates DNA synthesis by approximately 20%. This in vitro system appears to mimic normal in vivo viral DNA replication in all respects except for the initiation of new replicons (Anderson, Tapper, and DePamphilis, in preparation).

In view of the relatively small amount of ^{32}P label that appears in RNA-DNA linkages (10^{-5} to 10^{-6} of the *total* [α-^{32}P]dNTP substrate), we have critically examined the nearest neighbor analysis commonly employed to detect RNA-DNA covalent linkages in nascent DNA. Using this analysis, we have compared the rate at which nascent 4S DNA pieces are attached to growing daughter strands with the rate at which RNA-DNA covalent linkages disappear. Finally, we have studied whether the removal of RNA-DNA covalent linkages depends on concomitant DNA chain elongation. In a separate communication, we report the occurrence of an oligoribonucleotide of at least 7 residues covalently attached to the 5′ end of a fraction of the 4S nascent DNA ("Okazaki pieces") (Kaufmann et al., 1977).

Experimental Procedure

Cells and Virus. All experiments were done with a CV-1 African Green monkey kidney cell line obtained from P. Tegtmeyer, Department of Microbiology, New York University at Stony Brook. Cells were routinely grown at 37 °C in 100-mm diameter Lux dishes using Dulbecco-modified Eagle's medium supplemented with 5% fetal calf serum. Penicillin G, 500 units/mL, and streptomycin sulfate, 100 μg/mL, were added to virus-infected cells as a safeguard against bacterial contamination. The small plaque SV40 strain, Rh911 (Girardi, 1965), was grown by infection of MA134 cells (Microbiological Associates) at 0.01 or less plaque forming unit per cell. Virus was extracted 10 to 12 days later by scraping the infected cells into their medium with a rubber policeman and subjecting the suspension to three cycles of freezing in dry ice and ethanol bath and thawing at 30 °C. The cellular debris was removed by sedimentation at 10 000*g* for 10 min. The supernatant was adjusted to 10% in fetal calf serum before being stored in small aliquots at −35 °C.

Cytosol. Small amounts of cytosol were prepared from uninfected CV-1 cells as previously described (DePamphilis and Berg, 1975). All steps were carried out at 2 °C or on ice. Large amounts were prepared from 100 roller bottles by decanting the medium, washing the cells twice with 25 mL of 10 mM Hepes-Na, pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol, and then removing the cells with a rubber policeman. This resulted in a lysate which was centrifuged for 10 min at 5000*g* to remove the nuclei. The nuclear pellet was suspended in 200 mL of the same buffer, homogenized by three strokes of a loose fitting (pestle A) Dounce homogenizer, and again sedimented. The two supernatants were combined, adjusted to 0.1 M KCl, and centrifuged at 100 000*g* for 1 h. The soluble cytosol fraction was brought to 80% saturation with solid ammonium sulfate, and the precipitate was collected by centrifugation, resuspended, and dialyzed overnight against 20 mM Hepes-Na, pH 7.8, 50 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.4 M ethylene glycol. The cytosol fraction contained 10 to 15 mg of protein per mL and was stable for at least 2 months at −70 °C. This fraction contained protein factors required for DNA replication which presumably leaked out of the nuclei (DePamphilis and Berg, 1975).

Preparation of Nuclei from SV40-Infected Cells. CV-1 cells which had just reached confluency in 100-mm diameter plastic dishes were infected with SV40 virus at a multiplicity of 10 to 20 plaque-forming units per cell. Thirty-six hours after infection, when the rate of viral DNA synthesis had reached a maximum, the medium was decanted and the cell monolayers were washed twice with 5 mL of ice-cold 10 mM Hepes-Na, pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol. All further steps were carried out either at 4 °C or on ice. The cells were drained before removing them with a rubber policeman. Cell lysis was completed in a Dounce homogenizer with three strokes of the tight-fitting pestle (pestle B). The lysate was then diluted 10-fold with 10 mM Hepes-Na, pH 7.8, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.25 M sucrose, and 0.02% Triton X-100 to give a final volume of approximately 35 mL per 5 dishes of infected cells. The diluted lysate (35 mL) was layered over 10 mL of 20% Ficoll (w/v) in 10 mM Hepes-Na, pH 7.8, 1 mM $MgCl_2$, and 0.5 mM dithiothreitol and centrifuged in a Sorvall HB-4 rotor for 10 min at 4000*g* to pellet the nuclei. The supernatant, including most of the Ficoll, was removed by aspiration and the pellet was suspended in 45 mL of 10 mM Hepes-Na, pH 7.8, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.25 M sucrose. The suspension was centrifuged for 5 min at 2000*g* and the nuclei pellet resuspended in the same buffer to yield a final volume of 0.3 to 0.4 mL.

SV40 DNA Synthesis in Isolated Nuclei. Nuclei (20 μL), isolated from SV40-infected CV-1 cells, were incubated either with 70 μL of cytosol (nuclei + cytosol), or with 70 μL of 20 mM Hepes-Na, pH 7.8, 50 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.4 M ethylene glycol (Nuclei − cytosol), and 10 μL of an assay mix consisting of a buffered salts solution containing deoxyribo- and ribonucleoside triphosphates and an ATP-regenerating system. The *final concentrations* were 46 mM Hepes-Na, pH 7.8, 65 mM KCl, 4.6 mM $MgCl_2$, 1 mM sodium ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate (EGTA), 280 mM ethylene glycol, 5 mM potassium phosphoenolpyruvate, 30 μg/mL pyruvate kinase (stored in glycerol, not $(NH_4)_2SO_4$), 50 mM sucrose, 0.28 mM dithiothreitol, 2 mM rATP, 100 μM each of rGTP, rUTP, and rCTP, and 20 μM each of dATP, dGTP, dCTP, and dTTP except that radioactive nucleotides were present at 10 μM unless otherwise noted. [α-^{32}P]dNTPs were present at a specific radioactivity of 50 to 200 μCi/nmol; [^3H]dNTPs were present at a specific radioactivity of 2.5 μCi/nmol. Cytosol, nuclei, and assay mix were combined in that order at 0 °C, and DNA synthesis was started by incubating the reaction mixture in a 30 °C water bath. In pulse-chase experiments, 5 μL of a 20 mM solution of the appropriate unlabeled dNTP was added to begin the chase. DNA synthesis was terminated by the addition of 200 μL of 1.2% sodium dodecyl sulfate, 40 mM EDTA, and 20 mM Tris-HCl, pH 7.6.

Isolation of Viral DNA. SV40 DNA was extracted from the terminated reaction mixtures by adding 200 μL of 2.5 M NaCl, gently mixing to avoid shearing cellular DNA, and then storing the extract at 4 °C for at least 12 h (Hirt, 1967). Cellular DNA was pelleted by centrifuging the extract for 30 min at 23 000*g* (2 °C). The supernatant, containing the viral DNA, was layered onto a 5 to 15% linear neutral sucrose gradient formed over a 4-mL cushion of 50% sucrose in a Beckman SW41 tube. In addition to RNase-free sucrose (Schwarz/Mann), these gradients contained 10 mM Tris-HCl, pH 7.5, 1 M NaCl, and 2 mM EDTA. Gradients were centrifuged for

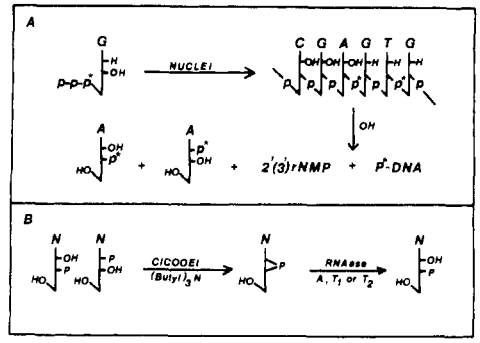

FIGURE 1: (A) Schematic representation of ^{32}P-label transfer from [α-^{32}P]dGTP to [2'(3')-^{32}P]rAMP upon alkaline hydrolysis of an RNA-DNA covalent linkage. (B) Conversion of a 2'(3')rNMP to a cyclic 2':3'-rNMP and hydrolysis of the cyclic nucleotide to yield a 3'-rNMP.

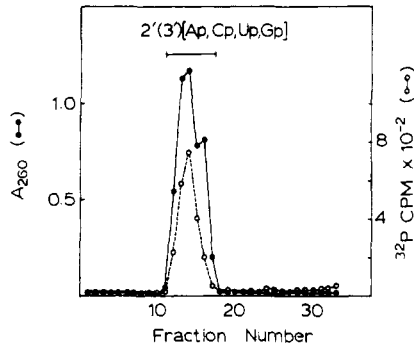

FIGURE 2: DEAE-Sephadex chromatography of alkali-treated SV40(RI) DNA. SV40(RI) [^{32}P]DNA, purified from nuclei labeled with [α-^{32}P]-dGTP for 1.5 min, was incubated in KOH and then chromatographed on DEAE-Sephadex to isolate the [2'(3')-^{32}P]rNMPs from [^{32}P]DNA. Details are found in Materials and Methods. Fractions 11-17 were pooled for chromatography on Bio-Rad AG1 resin (Figure 3). Similar results were obtained with SV40(RI) [^{32}P]DNA that had been labeled with [α-^{32}P]-dCTP, [α-^{32}P]dATP, or [α-^{32}P]dTTP.

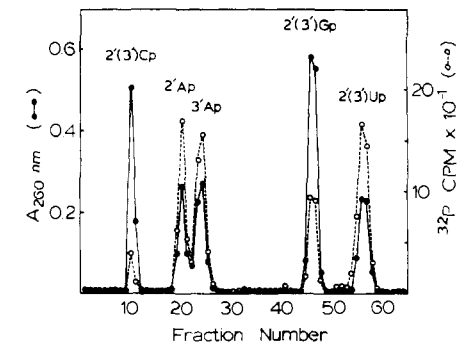

FIGURE 3: Bio-Rad AG1 chromatography of 2'(3')rNMPs. The [2'-(3')-^{32}P]rNMP pool (Figure 2) was chromatographed on Bio-Rad AG1 resin. The fractions corresponding to each [2'(3')-^{32}P]rNMP were pooled for further analysis. Similar results were obtained with SV40(RI) [^{32}P]DNA that had been labeled with [α-^{32}P]dCTP, [α-^{32}P]dATP, or [α-^{32}P]dTTP.

18 h at 37 000 rpm, 4 °C, collected through the bottom of the tube, and fractions containing [^{32}P]DNA sedimenting between 18 S and 28 S were pooled. This technique concentrated the SV40 (RI) DNA into a smaller volume than was possible using only a neutral sucrose linear gradient. This [^{32}P]DNA was shown to contain only SV40 DNA by sedimentation in alkaline sucrose gradients and electrophoresis in agarose gels (D. Tapper, unpublished results).

Alkaline Incubation of SV40(RI) [^{32}P]DNA. RNA-DNA covalent linkages were assayed quantitatively in SV40(RI) [^{32}P]DNA by incubating the sample in KOH to hydrolyze RNA and then isolating the [2'(3')-^{32}P]rNMPs that resulted from transfer of ^{32}P in rN-^{32}P-dN linkages (Figure 1A). SV40(RI) [^{32}P]DNA, isolated from a neutral sucrose gradient, was dialyzed against 10 mM Tris-HCl, pH 8.0, 0.4 M K_2HPO_4, 0.125 M potassium acetate, and 2 mM EDTA. Approximately 1-mL portions were incubated with 3 μL of 2 mg/mL salmon sperm DNA (Sigma, previously extracted with $CHCl_3$–isoamyl alcohol), 6 μL of 10 mg/mL yeast soluble RNA (Boehringer-Mannheim), and 30 μL of 1% hexadecyltrimethylammonium chloride (Eastman) for 30 min at 4 °C. The nucleic acid precipitate was collected in a 1.5-mL polypropylene Eppendorf microcentrifuge tube by centrifugation for 5 min, 12 000g, at 4 °C. The pellet was washed by centrifugation, first in 1 mL of cold 70% ethanol containing 0.1 M potassium acetate, then in 1 mL of cold absolute ethanol, and finally dried under vacuum. Alkaline hydrolysis of RNA was accomplished by dissolving the dried pellet in 60 μL of 0.3 N KOH and incubating for 20 h at 37 °C in a sealed Eppendorf tube. The total ^{32}P in the sealed tube was determined from Cerenkov radiation. The KOH was then neutralized with 0.8 mL of 30 mM acetic acid.

Isolation of [2'(3')-^{32}P]rNMPs. Following KOH hydrolysis and neutralization, the sample was diluted with 5 mL of water and adsorbed onto a 21 × 0.8 cm DEAE-Sephadex A-25 column previously equilibrated with 1 M sodium acetate, pH 5.0, and washed thoroughly with water. The rNMPs were separated from [^{32}P]oligodeoxyribonucleotides with a 160-mL linear gradient of 0 to 1 M triethylammonium acetate, pH 5.0, at a flow rate of 8 mL/h. Cerenkov radiation and A_{260} were measured in each 5-mL fraction. The 2'(3')rNMPs were identified as the major A_{260} peak(s) (Figure 2). When a large number of ^{32}P-transfer analyses were done, the DEAE-Sephadex columns (2–2.5-mL bed volume in disposable 5-mL pipettes) were eluted stepwise with triethylammonium acetate, pH 5.0, 0.025 to 0.5 M. The steps consisted of twenty 1.5-mL increments of 0.025 M triethylammonium acetate. These results were comparable to those obtained with a continuous linear gradient.

Separation of 2'(3')rNMPs. The 2'(3')rNMP pool from the DEAE-Sephadex column (Figure 2) was lyophilized to remove salt, dissolved in 70 mL of water, and adsorbed onto a Bio-Rad AG1-X8 column (27 × 0.8 cm) previously equilibrated with 1 M formic acid and thoroughly washed with water. Optimum separation of rNMPs was achieved by eluting with two consecutive 200-mL linear gradients; the first was 0 to 1 M formic acid and the second was 1 to 10 M formic acid (Figure 3). Fractions of 6 mL were collected at a flow rate of 13 mL/h at room temperature. No detectable radioactivity was found upon further elution with 1 N NaOH. The distribution of radioactivity in the four rNMPs was calculated from the radioactivity associated with each A_{260} peak.

Confirmation of the Identity of 2'(3')rNMPs. Individual rNMPs, isolated from a Bio-Rad AG1-X8 column, were lyophilized to remove formic acid and then converted to the sodium salt by lyophilization with equimolar amounts of $NaHCO_3$. The 2'(3')rNMPs were converted to the 2':3'-cNMPs by adding 5 μL of ethyl chloroformate and 15 μL of tri-*n*-butylamine to 50-μL samples and vortexing for 10 min in a sealed 1.5-mL Eppendorf tube (Michelson, 1959). The reaction mixture was then neutralized with 1 mL of 70 mM KH_2PO_4, pH 6.0, diluted with 10 mL of water, and adsorbed onto a DEAE-Sephadex A-25 column (9 × 0.6 cm) previously equilibrated with 1 M $NaHCO_3$ and washed extensively with water. The nucleotides were eluted in 20 steps consisting of

1.5-mL increments of 0.015 M NH_4HCO_3 from 0.115 M to 0.4 M. Each fraction was monitored for ^{32}P Cerenkov radiation and A_{260}, and the fractions containing 2′:3′-cNMPs were lyophilized to remove the salt. At neutral pH, cyclic nucleotides, with a charge of −1, were well resolved from 2′(3′)rNMPs with a charge of −2 (Figure 4). To further verify the identity of the 2′:3′-cNMPs, each cyclic nucleotide was converted into a 3′-rNMP by enzymatic hydrolysis. Cyclic nucleotides were dissolved in 1.2 mL of 0.2 M KH_2PO_4, pH 6.1, 5 mM EDTA, and RNase A (15 μL of 4 mg/mL) was added to cyclic UMP and cyclic CMP, RNase T1 (50 μL of 0.4 mg/mL) to cyclic GMP, and RNase T2 (50 μL of 0.4 mg/mL) to cyclic AMP. These solutions were incubated for 2 h at 30 °C and chromatographed as described above for cyclic nucleotides (Figure 4B).

Alkaline Sucrose Gradients. DNA was first dialyzed against 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, and 1 mM EDTA before layering 100-μL samples over 5 to 20% alkaline sucrose gradients. Sucrose gradients were formed in Beckman SW60 tubes from 5% sucrose containing 0.2 M NaOH and 0.8 M NaCl to 20% sucrose with 0.8 M NaOH and 0.2 M NaCl. In addition, the gradients contained 2 mM EDTA and 0.015% sodium dodecyl sarcosinate. Gradients were centrifuged for 6.5 h at 55 000 rpm (4 °C) and then collected dropwise from the bottoms of the tubes onto Whatman 3MM paper discs. The discs were dried and counted in a toluene-based scintillation fluid.

Reagents. Unlabeled nucleotides and nucleosides were purchased from either P-L Biochemicals or Sigma, the arabinofuranosyl analogues of dCTP and dATP from P-L Biochemicals, RNase A and T1 from Worthington Biochemicals, RNase T2 was from Sigma, [^{3}H]dATP and $^{32}P_i$ were from New England Nuclear, and [^{3}H]dTTP was from Schwarz/Mann. [α-^{32}P]dNTPs were prepared according to Symons (1974). 2′-Deoxyadenosine and 2′-deoxycytosine were chromatographed to remove any contaminating ribonucleosides (Dekker, 1965). In addition, all of the purified [α-^{32}P]dNTPs were treated with periodate (Wu, 1970) and then repurified prior to use.

Results

Detection of RNA-DNA Covalent Linkages in SV40(RI) [^{32}P]DNA. (i) *Release of [2′(3′)-^{32}P]rNMPs by KOH.* RNA-DNA covalent linkages can be detected by a form of nearest neighbor analysis using alkali or ribonucleases to specifically hydrolyze the RNA (Flügel and Wells, 1972; Wickner et al., 1972; Schekman et al., 1972). If DNA synthesis utilizes an RNA primer and [α-^{32}P]dNTPs, then a portion of the incorporated label should be recovered in 2′-(3′)rNMPs following alkaline incubation of the isolated DNA (Figure 1A). The amount of ^{32}P "transferred" to 2′(3′)rNMPs is a measure of the number of labeled phosphodiester linkages joining RNA to DNA.

SV40(RI) [^{32}P]DNA was purified from nuclei that had been briefly incubated in the presence of an [α-^{32}P]dNTP to label nascent DNA. Unlabeled tRNA was then added as an internal standard and the mixture incubated in 0.3 N KOH for 20 h at 37 °C to hydrolyze RNA. After neutralization the entire sample was chromatographed on DEAE-Sephadex to separate the 2′(3′)rNMPs from DNA (Figure 2). The internal RNA standard (A_{260}) was quantitatively converted to 2′-(3′)rNMPs. The fractions indicated in Figure 2 were pooled and subsequently analyzed by chromatography on Bio-Rad AG-1 resin. Greater than 96% of the ^{32}P-label chromatographed with the individual 2′(3′)rNMPs (Figure 3).

In order to identify the radioactive material associated with

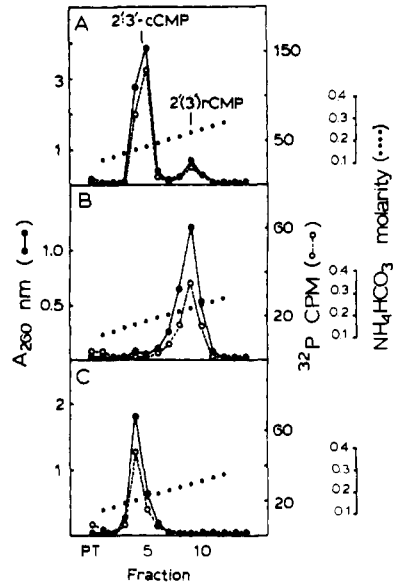

FIGURE 4: Formation and subsequent hydrolysis of cyclic 2′:3′-rCMP. The products obtained by alkali treatment of SV40(RI) [^{32}P]DNA pulse labeled with [α-^{32}P]dTTP were chromatographed on DEAE-Sephadex and Bio-Rad AG1 resin as shown in Figures 2 and 3. (A) Fractions from the AG1 chromatography containing 2′(3′)rCMP were pooled and treated with ethyl chloroformate as described in Materials and Methods. The resulting products were chromatographed on DEAE-Sephadex, and fractions 4 and 5, containing cyclic 2′:3′-rCMP, were pooled for further analysis. (B) Cyclic 2′:3′-rCMP was treated with RNase A (see Materials and Methods), and the products were chromatographed on DEAE-Sephadex using the same conditions as in panel A. (C) As a control, cyclic 2′:3′-rCMP was treated exactly as in B, but the RNase A was omitted.

the 2′(3′)rNMPs, each of the 2′(3′)rNMPs was chemically and enzymatically modified (Figure 1B). Individual 2′(3′)rNMPs were converted into cyclic 2′:3′-rNMPs by treatment with ethyl chloroformate in the presence of tri-*n*-butylamine (Michelson, 1959), and the resulting products purified by chromatography on DEAE-Sephadex (Figure 4A). The ^{32}P-labeled material chromatographed with the 2′:3′-cNMPs. Each of the four cyclic nucleotides was then treated with an appropriate RNase to convert it into a 3′-rNMP. cUMP and cCMP were treated with RNase A, cGMP was treated with RNase T1, and cAMP with RNase T2, and the products were again analyzed by chromatography on DEAE-Sephadex (Figure 4B). Again the ^{32}P-labeled material chromatographed with the A_{260} material. No hydrolysis of cyclic nucleotides was observed in the absence of added RNase (Figure 4C).

Throughout the above purification and analysis procedures, the ^{32}P-labeled materials consistently behaved as 2′(3′)rNMPs. Control experiments showed that these procedures easily distinguished 5′-dNMPs, 5′-rNMPs, and 3′-dNMPs from 2′-(3′)rNMPs. Therefore, we conclude that the ^{32}P-labeled compounds released by alkaline incubation of SV40(RI) [^{32}P]DNA were, in fact, [2′(3′)-^{32}P]rNMPs.

The same amount of ^{32}P-label transfer was also associated with SV40(RI) [^{32}P]DNA which had been purified on a CsCl-ethidium bromide density equilibrium gradient, whereas similarly purified SV40(I) [^{32}P]DNA, labeled either in vivo or in vitro, did not contain alkaline sensitive linkages and did not yield detectable ^{32}P transfer. SV40(RI) [^{32}P]DNA that was not incubated in KOH did not yield labeled nucleotides, demonstrating that none of the original labeled deoxyribonucleotides contaminated the purified DNA.

(ii) *[2′(3′)-^{32}P]rNMPs Originate from RNA-DNA Covalent linkages.* To exclude contaminating ^{32}P-labeled rNTPs, the [α-^{32}P]dNTPs were prepared from purified 2′-deoxyri-

TABLE I: Distribution of RNA-DNA Covalent Linkages in SV40(RI) DNA.[a]

[α-^{32}P]dNTP	[^{32}P]DNA (cpm)	Fraction of ^{32}P label transferred	Distribution of ^{32}P-label transfer (%)			
			rAMP	rCMP	rGMP	rUMP
dATP	6.45×10^6	0.0013	38 (36)	29 (27)	12 (16)	21 (22)
dCTP	8.85×10^6	0.0034	26 (27)	32 (25)	17 (23)	25 (25)
dGTP	1.74×10^6	0.0033	48 (37)	4 (3)	16 (24)	32 (37)
dTTP	2.45×10^6	0.0033	23 (24)	23 (24)	17 (17)	37 (35)

[a] Nuclei isolated from SV40-infected cells were incubated in the absence of cytosol for 1.5 min with [α-^{32}P]dATP, [α-^{32}P]dCTP, [α-^{32}P]dGTP, or [α-^{32}P]dTTP, as described in Materials and Methods. SV40(RI) [^{32}P]DNA was purified and then incubated with KOH; the resulting (2′-(3′)-^{32}P]rNMPs were analyzed by DEAE-Sephadex (Figure 2) and AG1 resin (Figure 3) chromatography. The total fraction of ^{32}P-label "transferred" was calculated by dividing the total ^{32}P-radioactivity in 2′(3′)rNMPs by the total ^{32}P-radioactivity incorporated into DNA. The distribution of ^{32}P-label among the four 2′(3′)rNMPs was calculated from the amount of ^{32}P-radioactivity associated with each 2′(3′)rNMP peak resolved by the AG1 chromatography (Figure 3). The numbers in parentheses represent the frequency of each rN-dN linkage as predicted from a nearest-neighbor analysis of SV40(I) DNA (J. Subak-Sharpe, unpublished data) and the assumption that RNA-DNA linkages are randomly distributed with respect to the SV40 genome.

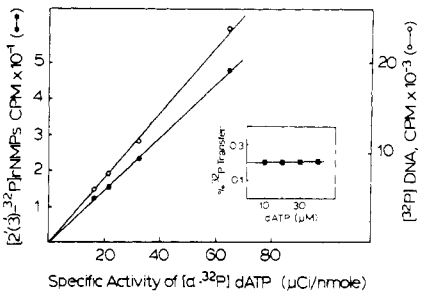

FIGURE 5: ^{32}P-label transfer as a function of [α-^{32}P]dATP specific radioactivity. Four samples of nuclei isolated from SV40-infected cells were incubated with 64 μCi of [α-^{32}P]dATP for 1.5 min at 30 °C as described in Materials and Methods. The specific radioactivity of the [α-^{32}P]dATP in each incubation was adjusted by prior addition of unlabeled dATP to the reaction tubes. The final specific radioactivities of [α-^{32}P]dATP were 64, 32, 21, and 16 μCi/nmol with final concentrations of dATP adjusted to 10, 20, 30, and 40 μM, respectively. SV40(RI) [^{32}P]DNA was isolated and analyzed for total incorporated ^{32}P radioactivity (○) as well as total ^{32}P-label transfer (●). Insert: The observed percent ^{32}P-label transfer, equal to (^{32}P cpm in 2′(3′)rNMPs)/(^{32}P cpm in DNA) × 100 (■), is plotted against the final concentration of dATP in the reactions.

bonucleosides and treated with periodate (Materials and Methods). However, to demonstrate directly that [2′(3′)-^{32}P]rNMPs received their ^{32}P label from [α-^{32}P]dNTPs incorporated into SV40(RI) DNA, the amount of ^{32}P appearing in rNMPs was compared with the specific radioactivity of the [α-^{32}P]dNTP. When the concentration of [α-^{32}P]dNTPs was saturating (10 μM or greater), the amount of ^{32}P label incorporated into DNA was proportional to the specific radioactivity of the substrate. Under these conditions the amount of ^{32}P label transferred to 2′-(3′)rNMPs was also proportional to the specific radioactivity of the [α-^{32}P]-dNTP substrate (Figure 5). Hence, the *percentage* of ^{32}P transferred to 2′(3′)rNMPs was independent of the specific radioactivity of the [α-^{32}P]dNTP substrate (Figure 5, insert). If, however, labeled RNA-RNA junctions, in addition to RNA-DNA junctions, had contributed [2′(3′)-^{32}P]rNMPs, then the percentage of ^{32}P-label transfer would have increased as the specific radioactivity of the [α-^{32}P]dNTP substrate was decreased by addition of unlabeled dNTP.[2] Moreover, no change in the amount of [2′(3′)-^{32}P]rNMPs was observed when the concentration of unlabeled rNTPs was varied in the DNA synthesis reaction mixture. Therefore, we conclude that the [2′(3′)-^{32}P]rNMPs received their ^{32}P label from the [α-^{32}P]dNTP substrate rather than from contaminating ^{32}P-labeled rNTPs.

The RNA-DNA covalent linkages present in SV40(RI) DNA were resistant to RNase T2 digestion at 4 °C in 1 M NaCl, presumably because they were present in a duplex structure. In a separate paper (Kaufmann et al., 1977), we show that these RNA-DNA covalent linkages are accounted for by the presence of oligoribonucleotides attached to the 5′ termini of nascent DNA chains.

Distribution of RNA-DNA Covalent Linkages in SV40(RI) DNA. All 16 possible RNA-DNA covalent linkages were found in SV40(RI) DNA (Table I). If rN-^{32}P-dN linkages were completely independent of the DNA template nucleotide sequence, then the total fraction of ^{32}P transferred should have been the same for each [α-^{32}P]dNTP. Although this was the case for dCTP, dGTP, and dTTP, the ^{32}P-label transfer from dATP was consistently two to three times less than expected. However, the fraction of ^{32}P-label transfer between a given dNTP and each of the four rNMPs was well correlated with the frequency of occurrence of the corresponding dN-dN linkages in SV40 DNA (Table I).

Transient Nature of RNA-DNA Covalent Linkages. Since RNA-DNA covalent linkages were found only in replicating SV40 DNA, they must be removed during the replication process. If these linkages result from RNA priming of nascent DNA, they should be removed at a rate equal to or faster than the rate at which nascent DNA chains are joined to growing daughter strands.

In the presence of cytosol, SV40 nascent DNA chains, mainly 50–150 nucleotides in length (4S DNA in alkaline sucrose gradients), are joined to longer growing daughter strands on SV40(RI) DNA. In order to quantitate this joining process, a pulse-chase experiment was performed. SV40(RI) DNA in isolated nuclei was labeled in the presence of cytosol with an [α-^{32}P]dNTP for 10–15 s, and then further incubated with an excess of unlabeled substrate for an additional 4.5 min. The labeled SV40(RI) DNA was analyzed by sedimentation in an alkaline sucrose gradient. During the chase more than 80% of the 4S DNA initially labeled (Figure 6A) was converted into longer strands (Figure 6B). When nascent DNA was pulse labeled for longer periods of time, its apparent rate of disappearance was faster presumably because those pieces of DNA most heavily labeled disappeared first. Similar pulse-chase experiments performed after an initial period of

[2] Observed % P* transfer = $[(\text{rNMP*})_{\text{RNA-DNA}} + (\text{rNMP*})_{\text{RNA-RNA}}]/[\text{P*} - \text{DNA}] = [K_1\ (\text{dNTP*/dNTP}) + (\text{rNMP*})_{\text{RNA-RNA}}]/K_2\ (\text{dNTP*/dNTP}) = (K_1/K_2) + [(\text{rNMP*})_{\text{RNA-RNA}}/K_2(\text{dNTP*})]\ (\text{dNTP}) = a + b(\text{dNTP})$, where K_1 and K_2 are the slopes for rNMP and DNA, respectively, in Figure 5, and a and b are constants for a given experiment.

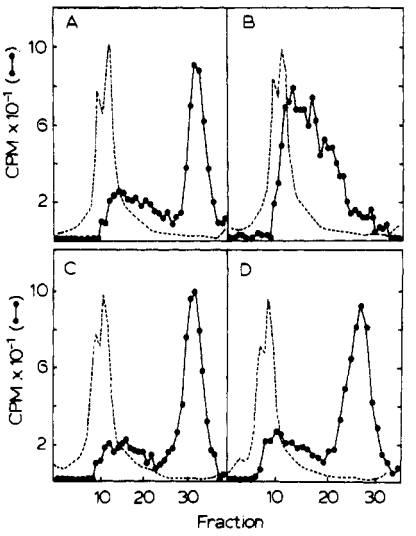

FIGURE 6: Alkaline sucrose gradient velocity sedimentation of SV40(RI) [^{32}P]DNA. Nuclei isolated from SV40-infected cells were incubated (with or without cytosol) for 1.3 min at 30 °C in the presence of unlabeled nucleotides and then 6 μCi of [α-^{32}P]dCTP was added as a pulse label to each sample and the incubation continued for 10 to 15 s. This short pulse-labeling time was necessary in order to obtain an approximate number-average measurement of the amount of 4S DNA chains. At the end of the pulse period, a 100-fold excess of unlabeled dCTP was added to each sample and the incubation was allowed to continue for various amounts of time. SV40(RI) [^{32}P]DNA was purified, precipitated with ethanol, and analyzed by sedimentation in an alkaline sucrose gradient as described in the Materials and Methods. SV40(II) [^{3}H]DNA (- - - -) was included in each gradient as an internal standard. The direction of sedimentation is from right to left. (A) Nuclei incubated in the presence of cytosol; no chase. (B) Nuclei incubated in the presence of cytosol; 4.5-min chase. (C) Nuclei incubated in the absence of cytosol; no chase. (D) Nuclei incubated in the absence of cytosol; 4.5-min chase. The proportion of 4S DNA chains present was estimated by integrating the areas under the peaks.

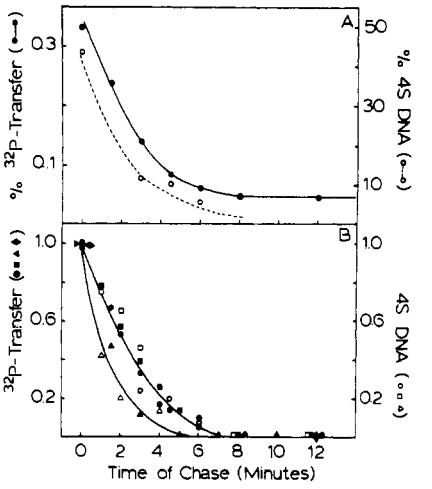

FIGURE 7: Removal of RNA-DNA covalent linkages in the presence of cytosol. (A) Nuclei isolated from SV40-infected cells were labeled for 1.5 min with [α-^{32}P]dCTP and then incubated with a 100-fold excess of unlabeled dCTP. The SV40(RI) [^{32}P]DNA was isolated and assayed for ^{32}P-label transfer (●). In a parallel experiment, nuclei in the presence of cytosol were pulse labeled, and the label was chased, as described in the legend to Figure 6. SV40(RI) [^{32}P]DNA purified from these nuclei was assayed for ^{32}P-labeled 4S DNA (○) by alkaline sucrose gradient sedimentation. (B) Data from several experiments were corrected for background and normalized as a fraction of the value observed with zero time of chase. One line was plotted through the ●, ○, ■, □, ♦ points and one line through the ▲, △ points. Actual values for % ^{32}P-label transfer at zero time and backgrounds (> 12 min) were as follows [(symbols) substrate, % ^{32}P-label transfer (at 0 and 12 min, respectively)]: (●, ○) [α-^{32}P]dCTP, 0.33 and 0.04; (■, □) [α-^{32}P]dATP, 0.18 and 0.02; (▲, △) [α-^{32}P]dTTP, 0.26 and 0.02; (♦) [α-^{32}P]dGTP, 0.52 and 0.23. Closed symbols represent ^{32}P-label transfer; open symbols represent 4S DNA assayed in parallel experiments. Note: The [α-^{32}P]dATP data were corrected for a secondary source of label transfer (see text) which caused the background to increase with apparent first-order kinetics from 0.02% to 0.09% during the chase.

DNA synthesis exhibited identical kinetics of 4S DNA joining. This demonstrated that during the first 5–10 min of SV40 DNA replication in vitro the process of DNA chain elongation approximates a steady state.

An analogous pulse-chase experiment was performed to quantitate the rate at which RNA-DNA covalent linkages were removed from SV40(RI) DNA during replication. SV40(RI) [^{32}P]DNA, labeled in vitro with [α-^{32}P]dCTP for 1.5 min, was further incubated with an excess of unlabeled dCTP. SV40(RI) [^{32}P]DNA, chased in this manner for various times in vitro, was isolated and analyzed for the presence of rN-^{32}P-dN linkages (Figure 7A). More than 85% of the labeled RNA-DNA junctions initially present in SV40(RI) [^{32}P]DNA disappeared during an 8-min chase. The disappearance of RNA-DNA covalent linkages paralleled the conversion of 4S DNA to longer strands. Similar pulse-chase experiments were performed with [α-^{32}P]dATP, [α-^{32}P]-dGTP, or [α-^{32}P]dTTP (Figure 7B). These data do not define a single line because of variation in the activity of the different cytosol preparations used in the experiments. However, *within a given experiment* the rate of disappearance of RNA-DNA covalent linkages was the same as the rate of conversion of nascent 4S DNA into longer DNA strands.

In most pulse-chase experiments the level of ^{32}P-label transfer decreased 80 to 90% within 6 to 8 min (Figures 7 and 8). The remaining rN-^{32}P-dN linkages persisted for 20 min or more and may have resulted from either abortive DNA replication events or reactions unrelated to DNA replication involving enzymes capable of forming rN-dN linkages. Examples of such enzymes are polynucleotide phosphorylase (Kaufmann and Littauer, 1969), terminal transferase (Roychoudhury and Kössel, 1971), RNA polymerase (Hurwitz et al., 1972), and mammalian DNA ligase (Bedows et al., 1977).

Pulse-chase experiments using [α-^{32}P]dATP revealed an anomaly not observed with the other labeled substrates. Although the initial rate of removal of rN-dA linkages was equivalent to that found for the other rN-dN linkages, the number of rN-dA linkages began to increase again with apparent first-order kinetics after 8 min of incubation in a 100-fold excess of unlabeled dATP. This "new" ^{32}P-label transfer was mainly to rAMP and was found in polynucleotide chains of greater than 400 residues. A similar phenomenon has been reported for rN-dA linkages in lymphocyte lysates (Tseng and Goulian, 1975a). This anomaly was *not* observed when cytosol was absent, although rN-dA linkages were still removed under these conditions (Figure 8). Thus, there appears to be a secondary source of rN-^{32}P-dA linkages unrelated to RNA priming of nascent DNA.

Removal of RNA-DNA Covalent Linkages in the Absence of 4S DNA Joining. In the absence of cytosol, nuclei from SV40-infected cells support only limited DNA synthesis and are unable to join nascent 4S DNA chains to growing daughter strands in SV40(RI) DNA (Figures 6C and 6D; DePamphilis and Berg, 1975). To determine whether this defect in DNA joining resulted from a failure to remove RNA primers, a pulse-chase experiment was performed in the absence of cytosol. Nuclei were briefly labeled with [α-^{32}P]dCTP and then incubated in excess unlabeled dCTP exactly as described above

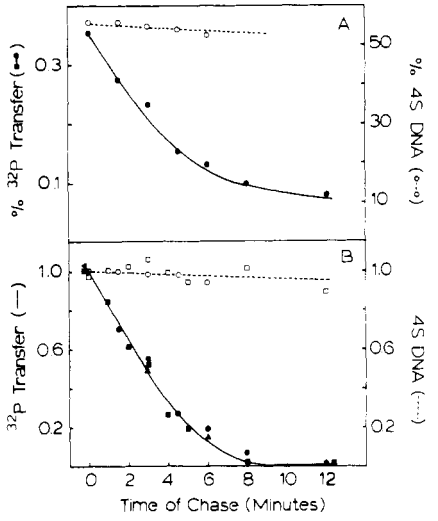

FIGURE 8: Removal of RNA-DNA covalent linkages in the absence of cytosol. (A) Nuclei isolated from SV40-infected cells were pulse-labeled with [α-^{32}P]dCTP, incubated with excess unlabeled dCTP, and the SV40(RI) [^{32}P]DNA analyzed for ^{32}P-label transfer (●) and 4S DNA (○) as described in Figure 7A. However, cytosol was not present during the incubations. (B) Data from different experiments done in the absence of cytosol were corrected for background and normalized as a fraction of the value observed with zero time of chase. Actual values for % ^{32}P-label transfer at zero time and backgrounds (>12 min) were as follows [(symbols) substrate, % ^{32}P-label transfer (at 0 and 12 min, respectively)]: (●, ○) [α-^{32}P]dCTP, 0.36 and 0.04; (■, □) [α-^{32}P]dATP, 0.20 and 0.04; (▲) [α-^{32}P]dTTP, 0.35 and 0.04. Closed symbols represent ^{32}P-label transfer; open symbols represent 4S DNA measurements made in parallel experiments.

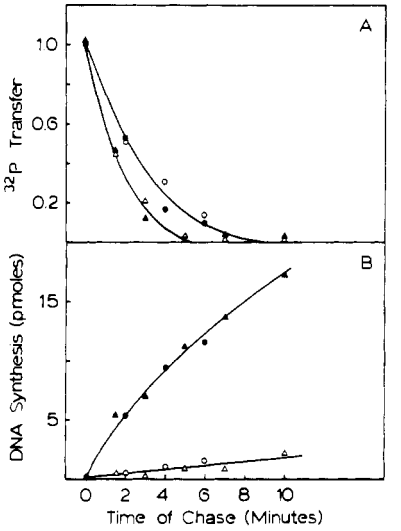

FIGURE 9: Effects of *ara*-CTP and *ara*-ATP on the removal of RNA-DNA covalent linkages. Nuclei isolated from SV40-infected cells were incubated in the presence of cytosol for 1.5 min at 30 °C either with [α-^{32}P]dTTP and [^{3}H]dATP (when *ara*-CTP was used), or with [α-^{32}P]-dCTP and [^{3}H]dTTP (when *ara*-ATP was used). Samples were then incubated with a 100-fold excess of either dTTP or dCTP, respectively. At the start of the chase period, 20 nmol of either *ara*-CTP (with the dTTP) or *ara*-ATP (with the dCTP) was added; control samples did not receive any inhibitor. SV40(RI) [^{3}H, ^{32}P]DNA was purified, and an aliquot was withdrawn for measuring acid-precipitable radioactivity. The remainder was analyzed for ^{32}P-label transfer. Acid-precipitable radioactivity was measured by adding to the aliquot 25 μg of salmon sperm carrier DNA and precipitating the [^{3}H, ^{32}P]DNA with cold 1 N HCl containing 0.5% sodium pyrophosphate. Precipitates were collected onto GF/C filters, washed three times with 6 mL of 1 N HCl, 0.5% sodium pyrophosphate, and once with 95% ethanol. Dried filters were counted in a toluene-based scintillation fluid. (A) ^{32}P-label transfer data were corrected for background and normalized as a fraction of the value observed with zero time of chase. Actual values for ^{32}P-label transfer at zero time and backgrounds were as follows [(symbol) substrate, inhibitor, % ^{32}P-label transfer (for zero time and background, respectively)]: (●) [α-^{32}P]dCTP, none, 0.44 and 0.05; (○) [α-^{32}P]dCTP, *ara*-ATP, 0.44 and 0.10; (▲) [α-^{32}P]dTTP, none, 0.45 and 0.16; (△) [α-^{32}P]dTTP, *ara*-CTP, 0.45 and 0.21. Separate lines are drawn through the points representing each [α-^{32}P]dNTP. (B) The amount of DNA synthesized during the chase period, in the presence or absence of inhibitors, was calculated from the total amounts of ^{3}H radioactivity incorporated into SV40(RI) DNA. [^{3}H]dTTP, no *ara*-ATP (●); [^{3}H]dTTP, plus *ara*-ATP (○); [^{3}H]dATP, no *ara*-CTP (▲); [^{3}H]dATP, plus *ara*-CTP (△).

except that cytosol was omitted. The results (Figure 8A) showed that the initial synthesis and disappearance of RNA-DNA covalent linkages were comparable to those observed in the presence of cytosol (Figure 7A). Similar pulse-chase experiments done using [α-^{32}P]dATP or [α-^{32}P]dTTP yielded identical results (Figure 8B). Thus, isolated nuclei retained the ability to remove RNA-DNA covalent linkages even though they were unable to convert nascent 4S DNA into longer strands.

Irrespective of the presence or absence of cytosol, similar *percentages* of ^{32}P-label transfer were obtained from SV40(RI) [^{32}P]DNA labeled for 1.5 min with an [α-^{32}P]-dNTP. In the presence of cytosol, however, the total *amounts* of ^{32}P incorporation and ^{32}P-label transfer were 1.5 to 2 times greater. The removal of RNA-DNA covalent linkages was also 1.3 to 2 times faster, on a percentage basis, in the presence of cytosol (Figures 7 and 8). This means that RNA-DNA covalent linkages actually disappear 2 to 3 times faster in the presence of cytosol than in its absence. Hence, although cytosol is not required to remove RNA-DNA covalent linkages from SV40(RI) DNA, its presence stimulates this process.

Removal of RNA-DNA Covalent Linkages during Inhibition of DNA Synthesis. Arabinosyl analogues of deoxyribonucleotides are strong inhibitors of in vitro eukaryotic DNA replication (Hunter and Francke, 1974b, 1975). Two of these analogues, *ara*-ATP and *ara*-CTP, were tested for their effects on the removal of RNA-DNA covalent linkages from SV40(RI) DNA in vitro. Nuclei were isolated from SV40-infected cells, briefly labeled with [α-^{32}P]dCTP in the presence of cytosol, and then incubated with an excess of unlabeled dCTP and 200 μM *ara*-ATP. [^{3}H]dTTP was present during both the pulse and chase to continuously monitor DNA synthesis. SV40(RI) [^{3}H, ^{32}P]DNA was isolated and assayed for ^{32}P-label transfer and ^{3}H incorporation. A similar experiment was also done with [α-^{32}P]dTTP as the pulse label, [^{3}H]dATP as the continuous label, and *ara*-CTP as the inhibitor.

When either *ara*-ATP or *ara*-CTP was used at a concentration of 200 μM, the RNA-DNA linkages disappeared at a rate comparable to that observed in the controls (Figure 9A) despite a 10-fold reduction in the rate of DNA synthesis (Figure 9B). The amount of ^{32}P-labeled 4S DNA converted into longer strands decreased 4-fold (not shown). The same results were obtained with nuclei incubated in the absence of cytosol except that all of the ^{32}P-labeled 4S DNA was prevented from joining to longer strands. Therefore, DNA synthesis does not appear to be necessary for the removal of RNA-DNA covalent linkages from SV40(RI) DNA to occur.

For both *ara*-ATP and *ara*-CTP the inhibition of DNA synthesis was competitive with substrate and became progressively more severe at higher concentrations (Table II). *ara*-ATP had little effect on the removal of RNA-DNA covalent linkages at any concentration. However, at intermediate concentrations of *ara*-CTP, unusually high levels of ^{32}P-label transfer were present at the end of the chase. This anomalous effect of intermediate *ara*-CTP concentrations was also ob-

served when a pulse-chase experiment was performed using [α-^{32}P]dGTP as the label donor (not shown). Preliminary data suggest that *ara*-NTP analogues may, under some conditions, give rise to spurious ^{32}P-label transfer contributions from sources other than replicating DNA. Studies are currently under way to clarify the nature of these sources.

Discussion

RNA-DNA covalent linkages in SV40(RI) DNA were quantitated by measuring the release of [2′(3′)-^{32}P]rNMPs from alkali-treated [^{32}P]DNA. These linkages are accounted for by the existence of uniquely sized oligoribonucleotides, at least 7 residues long, attached to the 5′ termini of nascent SV40 DNA chains (Kaufmann et al., 1977). Similar structures have previously been observed in replicating polyoma DNA and have been given the name "initiator RNA" (iRNA) (Reichard et al., 1974).

All 16 possible rN-dN dinucleotide sequences were detected in SV40(RI) DNA (Table I). This is in accord with ^{32}P-label transfer results from other in vitro eukaryotic DNA replication systems, including nuclei from polyoma-infected mouse cells (Magnusson et al., 1973; Hunter and Francke, 1974a), lysates of CHO cells (Waqar and Huberman, 1975a), lysates of human lymphocytes (Tseng and Goulian, 1975a), and intact plasmodia of *Physarum polycephalum* (Waqar and Huberman, 1975b). Table I shows that the rN-dN linkage frequencies in nascent SV40(RI) DNA were well correlated with the corresponding dN-dN linkage frequencies in SV40(I) DNA. The frequencies of rN-dC and rN-dA linkages in nascent DNA chains from native SV40(RI) DNA were the same as those frequencies found in subgenome fractions of nascent DNA defined by their ability to anneal to specific SV40 restriction fragments (Kaufmann et al., 1977). Moreover, RNase A digestion of initiator RNA from polyoma(RI) DNA did not reveal a unique sequence (Reichard et al., 1974). Taken together, these data show that such RNA-DNA covalent linkages are not synthesized at unique nucleotide sequences in the template DNA strand.

If RNA-DNA covalent linkages occurred randomly with respect to nucleotide sequence, then, regardless of base composition, the total fraction of ^{32}P-label transferred from each [α-^{32}P]dNTP should have been the same. In SV40(RI) DNA the rN-dC, rN-dG, and rN-dT linkage groups were found in equivalent amounts, but the rN-dA linkages were consistently two to three times less prevalent. In polyoma(RI) DNA both rN-dA and rN-dT linkages are two to three times less frequent than rN-dC or rN-dG linkages (Magnusson et al., 1973; Hunter and Francke, 1974a). Nascent DNA in mammalian cells exhibits approximately equal frequencies of each RNA-DNA linkage group (Waqar and Hubernan, 1975a; Tseng and Goulian, 1975a), while nascent DNA in *Physarum* contains rN-dG linkages about one order of magnitude more frequently than the other three linkage groups (Waqar and Huberman, 1975b). Therefore, some rN-dN linkage groups may be either preferentially synthesized or preferentially degraded.

When nuclei synthesized DNA in the presence of cytosol, the rate at which RNA-DNA covalent linkages disappeared was equal to the rate at which 4S DNA chains were converted to longer strands (Figure 7). This suggests that removal of an RNA primer may be the rate-limiting step during the normal process of joining 4S DNA chains in vitro. A transient nature for RNA-DNA linkages has also been found in polyoma (Pigiet et al., 1974) and lymphocyte (Tseng and Goulian, 1975a) in vitro DNA replication systems, although in these studies the rate of removal of RNA-DNA linkages was not compared directly with the rate of disappearance of short pieces of nascent DNA.

TABLE II: Effect of *ara*-ATP and *ara*-CTP Concentrations on the Removal of RNA-DNA Covalent Linkages.[a]

Time of chase (min)	*ara*-ATP (μM)	DNA synthesis (pmol)	Fraction of ^{32}P-label transfer
0	0	0	1.00
6	0	11.6	0
6	10	10.8	0.22
6	50	3.9	0.21
6	200	1.6	0.12
6	100 + 1 mM dATP	9.5	0.17
Time of chase (min)	***ara*-CTP (μM)**	**DNA synthesis (pmol)**	**Fraction of ^{32}P-label transfer**
0	0	0	1.00
10	0	17.3	0
10	10	13.7	1.55
10	50	4.7	0.81
10	200	2.1	0.12
10	100 + 1 mM dCTP	16.8	0.47

[a] Nuclei isolated from SV40-infected cells were pulse labeled in the presence of cytosol with [α-^{32}P]dCTP or [α-^{32}P]dTTP and then incubated with either excess dCTP and *ara*-ATP for 6 min or excess dTTP and *ara*-CTP for 10 min, as described in Figure 9. The concentration of the arabinosyl nucleotide was varied in each experiment. ^{32}P-label transfer and DNA synthesis were measured as described in Figure 9. ^{32}P-label transfer values were corrected for background and normalized as a fraction of the value observed with zero time of chase.

RNA-DNA covalent linkages also were removed in the absence of cytosol, a condition where joining of 4S DNA chains was strongly inhibited (Figures 6 and 8). Moreover, when the rate of DNA synthesis was inhibited 90% with either *ara*-CTP or *ara*-ATP, removal of RNA-DNA covalent linkages still occurred at a rate similar to that of controls (Figure 9). In contrast to our results, Tseng and Goulian (1975a,b) reported that the removal of RNA-DNA covalent linkages was inhibited by the absence of cytosol or the presence of *ara*-CTP. The apparent cytosol requirement they observed may have been due to the 10-fold higher concentration of detergent used in preparing their lymphocyte nuclei. This may have resulted in the extraction of factors required for the removal of RNA-DNA linkages as well as factors required for the joining of nascent 4S DNA chains. Their result using 50 μM *ara*-CTP may be compared with the unusually high ^{32}P-label transfer values we observed in the presence of *ara*-CTP in this concentration range (Table II). However, at higher concentrations *ara*-CTP does *not* appear to inhibit RNA-DNA linkage removal in our system, nor does *ara*-ATP appear to inhibit linkage removal at *any* concentration, although it inhibits DNA synthesis as effectively as *ara*-CTP. We have no explanation at present for this anomalous effect of *ara*-CTP on ^{32}P-label transfer measurements.

Thus, under optimal conditions, it appears that the processes of RNA primer excision and DNA chain elongation are closely coordinated, although they may be uncoupled when 4S DNA joining or DNA synthesis is arrested. These results also imply that excision of RNA primers in mammalian nuclei does not occur via a 5′- to 3′-exonuclease activity tightly complexed to a DNA polymerase activity (e.g., DNA polymerase I from *E. coli*) as is thought to be the case in prokaryotes (Kornberg, 1974; Lehman and Uyemura, 1976). However, if an RNase

H is involved in removing RNA primers, a second activity must accompany it to remove the rN-dN linkages since no known RNase H enzyme can accomplish this (Stavrianopoulos et al., 1976; Berkower et al., 1973).

Acknowledgments

We are indebted to Dr. John Subak-Sharpe for providing us with his most recent data on nearest neighbor analysis of SV40 DNA and to the M.I.T. Cell Culture Center for growing large quantities of CV-1 cells to use in preparing cytosol.

References

Bedows, E., Wachsman, J. T., and Gumport, R. I. (1977), *Biochemistry 16,* 2231-2235.

Berkower, I., Leis, J., and Hurwitz, J. (1973), *J. Biol. Chem. 248,* 5914-5921.

Dekker, C. A. (1965), *J. Am. Chem. Soc. 87,* 4027-4029.

DePamphilis, M. L., Beard, P., and Berg, P. (1975), *J. Biol. Chem. 250,* 4340-4347.

DePamphilis, M. L., and Berg, P. (1975), *J. Biol. Chem. 250,* 4348-4354.

Flügel, R. M., and Wells, R. D. (1972), *Virology 48,* 394-401.

Girardi, A. J. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54,* 445-450.

Hirt, B. (1967), *J. Mol. Biol. 26,* 365-369.

Hunter, T., and Francke, B. (1974a), *J. Mol. Biol. 83,* 123-130.

Hunter, T., and Francke, B. (1974b), *J. Virol. 13,* 125-139.

Hunter, T., and Francke, B. (1975), *J. Virol. 15,* 759-775.

Hurwitz, J., Yarbrough, L., and Wickner, S. (1972), *Biochem. Biophys. Res. Commun. 48,* 628-635.

Kaufmann, G., Anderson, S., and DePamphilis, M. L. (1977), *J. Mol. Biol.* (in press).

Kaufmann, G., and Littauer, U. Z. (1969), *FEBS Lett. 4,* 79-83.

Kornberg, A. (1974), DNA Synthesis, San Francisco, Calif., W. H. Freeman.

Kornberg, A. (1976), in RNA Polymerase, Losick, R., and Chamberlin, M., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory, pp 331-352.

Lehman, I. R., and Uyemura, D. G. (1976), *Science 193,* 963-969.

Magnusson, G., Pigiet, V., Winnacker, E. L., Abrams, R., and Reichard, P. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 412-415.

Michelson, A. M. (1959), *J. Chem. Soc.,* 3655-3669.

Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., Kainuma, R., Sugino, A., and Iwatsuki, N. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33,* 129-142.

Pigiet, V., Elliasson, R., and Reichard, P. (1974), *J. Mol. Biol. 84,* 197-216.

Reichard, P., Elliasson, R., and Söderman, G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4901-4905.

Roychoudhury, R., and Kössel, H. (1971), *Eur. J. Biochem. 22,* 310-320.

Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L. L., and Kornberg, A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2691-2695.

Stavrianopoulos, J. G., Gambino-Giuffrida, A., and Chargaff, E. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1087-1091.

Symons, R. H. (1974), *Methods Enzymol. 29,* 102-115.

Tseng, B. Y., and Goulian, M. (1975a), *J. Mol. Biol. 99,* 339-346.

Tseng, B. Y., and Goulian, M. (1975b), *J. Mol. Biol. 99,* 317-337.

Waqar, M. A., and Huberman, J. A. (1975a), *Cell 6,* 551-557.

Waqar, M. A., and Huberman, J. A. (1975b), *Biochim. Biophys. Acta 383,* 410-420.

Wickner, W., Brutlag, D., Schekman, R., and Kornberg, A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 965-969.

Wu, R. (1970), *J. Mol. Biol. 51,* 501-521.