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Synthesis of Simian Virus 40 DNA in Isolated Nuclei

(discontinuous synthesis/4S fragments/DNA maturation)

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ABSTRACT **Nuclei isolated from African green monkey kidney cells infected with simian virus 40 at different times after infection maintain *in vitro* the same temporal sequence of host and viral DNA synthesis as that seen in intact cells. The viral DNA synthesized by the nuclei of cells previously infected for 32–35 hr was characterized by centrifugation through neutral and alkaline sucrose gradients, and by isopycnic banding in a propidium iodide–cesium chloride gradient. DNA synthesis in this system is maintained for only 4–5 min. Neutral sucrose gradient analysis showed that most of the radioactivity is associated with the replicative intermediate of simian virus 40 DNA and the rest sediments at 5–7 S. Alkaline gradient analysis showed that 50–60% of the radioactivity sediments as 3–7S fragments, and the rest between 7 and 16 S. Pulse-chase experiments showed that in this system 3–7S fragments do not mature into long chains. A model is presented to explain the failure of these fragments to join into long chains in this *in vitro* nuclear system.**

Isolated cell nuclei capable of synthesizing DNA *in vitro* provide a good system for studying the process of DNA replication in mammalian cells and control mechanisms that regulate DNA synthesis. Nuclear DNA-synthesizing systems from mammalian cells, in which meaningful DNA replication can be studied, have been described (1–4). Polyoma DNA replication in isolated nuclei from infected cells has also been recently described (5, 6).

Studies on the replication of simian virus 40 (SV40) DNA *in vivo* have shown that the DNA synthesis in SV40 is discontinuous (7) and bidirectional (8, 9). Replication involves the synthesis of 4S fragments of DNA which are then joined to growing chains. Involvement of 4S fragments has also been demonstrated in polyoma DNA replication during DNA synthesis *in vitro* in isolated nuclei (10). Inhibition of DNA synthesis *in vivo* by 5-fluorodeoxyuridine or hydroxyurea (11, 12) results in the accumulation of 4S fragments of DNA.

This communication describes a nuclear SV40 DNA replication system synthesizing 3–7S fragments which do not mature into long chains. A proportion of these DNA fragments is associated with the replicating SV40 DNA molecules. In this system the steps of DNA maturation are dissociated, facilitating the characterization of the sequential events of the maturation process.

MATERIALS AND METHODS

Cells. Secondary cultures of African green monkey kidney (sec. AGMK) cells were made from primary monolayers supplied in 32-oz (947-ml) bottles by North American Biologicals, Rockville, Md. Monolayers were trypsinized and 3 to 5 × 10^5 cells were plated on 150-mm plastic petri dishes (Falcon Plastics) in Eagle's medium supplemented with fetal-calf serum (final concentration 5%, Flow Laboratories, Inc.) and glutamine (2 mM). Cells were fed again 3–4 days after planting and became confluent within 7–8 days. Monolayers were inoculated with virus 4–7 days after they had become confluent (1.0 to 1.5 × 10^7 cells per 150-mm plate).

Virus. A strain of SV40 that forms small plaques was obtained from N. Salzman. Stock virus was made by one passage on Vero cells at low multiplicity of infection (0.1 plaque-forming unit per cell). This stock virus was used for all the experiments.

Infection of AGMK Cells with SV40. Medium was removed from confluent sec. AGMK cells contained in 150-mm plastic dishes and cells were infected by the addition of 5 ml of virus inoculum (about 50 plaque-forming units per cell). Plates were incubated for 30 min at 37° in a CO_2 incubator and gently rocked every 10 min during this period. At the end of the adsorption period, 30 ml of Eagle's medium containing fetal-calf serum (5%) and glutamine (2 mM) was added to each plate. Mock-infected cells were treated exactly the same way except that they received 5 ml of Eagle's medium instead of virus inoculum during the adsorption period.

Isolation of Nuclei. The medium was removed from the cell monolayer in a 150-mm petri dish and washed three times with 5-ml portions of ice-cold phosphate-buffered saline (pH 7.0). Nuclei were isolated at 4°. The monolayer was washed with 3 ml of ice-cold lysing buffer [0.01 M phosphate buffer, pH 7.5, containing $MgCl_2$ (2 mM) and dithiothreitol (1 mM)]. The cells were then covered with 3 ml of the same buffer and detached with a "rubber policeman." The detached cells were poured into a loose-fitting Vitro Dounce homogenizer and the plate was washed with an additional 2 ml of lysing buffer. After 8 to 10 strokes in the homogenizer, 0.1 ml of 5% Triton X-100 was added to give a final concentration of 0.1%. After eight more strokes, 0.9 ml of 2 M sucrose was added to give a final concentration of 0.3 M sucrose. The suspension was centrifuged for 20 min at 800 × *g*. The nuclear pellet was resuspended in Isotonic buffer [50 mM Tris, pH 8.0, containing sucrose (0.2 M), EDTA (0.1 mM), $MgCl_2$ (1 mM), and dithiothreitol (2 mM)], at a final volume of 0.25 ml. An aliquot of this nuclear suspension was precipitated with trichloroacetic acid and DNA was estimated by the Burton method (13).

Conditions of DNA Synthesis in Vitro with the Nuclei. Each incubation mixture contained 0.2 ml of nuclear suspension which contained 6 to 9 × 10^6 nuclei and 200–300 μg of DNA.

Abbreviations: SV40, simian virus 40; AGMK cells, African green monkey kidney cells.

Nuclei were incubated at 37° in a final volume of 0.25 ml in 80 mM Tris buffer, pH 7.9, containing dATP, dGTP, dCTP, rCTP, rUTP, and rGTP (each at 0.1 mM), ATP (2.4 mM), [^{3}H]dTTP (0.01 mM, 15,000 cpm/pmol, determined on nitrocellulose filters), $MgCl_2$ (5.8 mM), NaCl (8 mM), sucrose (360 mM), dithiothreitol (2.6 mM), $CaCl_2$ (0.4 mM), EDTA (0.08 mM), phosphoenol pyruvate (5 mM), and pyruvate kinase (16 μg/ml). At the end of incubation, 0.1 ml of 2.1% sodium dodecyl sulfate solution was added (final concentration, 0.6%) to lyse the nuclei. The DNA was then fractionated as described by Hirt (14). After 30 min at room temperature, 0.1 ml of 4.5 M NaCl was added (final concentration, 1 M). The tubes were inverted gently several times to effect mixing and then kept overnight at 4°. The mixture was centrifuged for 45 min at 3500 rpm in a no. 295 rotor in an International PR-2 Centrifuge with the high-speed attachment (18,000 × *g*). The supernatant fluid was referred to as "Hirt supernatant" and the pellet fraction as "Hirt pellet." The Hirt supernatant was dialyzed for 48 hr against 0.01 M Tris, pH 7.3, containing NaCl (0.05 M) and EDTA (0.005 M). The dialysis buffer was changed after every 12 hr. Radioactivity was measured by precipitating an aliquot with 10% trichloroacetic acid containing thymidine (1 mM) and Na-pyrophosphate (50 mM) and filtering through Millipore nitrocellulose filters, which were then washed with 5% trichloroacetic acid–thymidine-pyrophosphate and with chloroform–methanol 1:1 mixture. Hirt pellets were dissolved overnight in 0.5 ml of 0.5 M NaOH at 37°. Aliquots were precipitated and filtered through Whatman GF/c filters and washed as above. Nitrocellulose filters were dissolved in 1 ml of Cellosolve, and radioactivity counted in toluene-Triton X-100 scintillation fluid (15). GF/c filters were counted in toluene-base scintillation fluid.

Gradient Analysis. Dialyzed Hirt supernatants were sedimented in 5–30% neutral sucrose gradients containing NaCl (1 M), Tris (0.01 M, pH 7.2), and EDTA (0.01 M) in a Spinco SW41 rotor at 26,000 rpm and 10° for 16 hr with ^{14}C-labeled SV40 components I and II as markers (15), which give peaks at 21 and 16 S, respectively. Samples were also sedimented in 10–30% alkaline sucrose gradients containing Tris (0.01 M), NaCl (0.7 M), NaOH (0.3 M), EDTA (0.001 M), and Sarkosyl (0.015%) in an SW41 rotor at 40,000 rpm and 10° for 17½ hr with ^{14}C-labeled SV40 component II as marker (15), which gives peaks at 18 and 16 S. Fractions were counted directly in a toluene–Triton X-100 scintillation fluid (15).

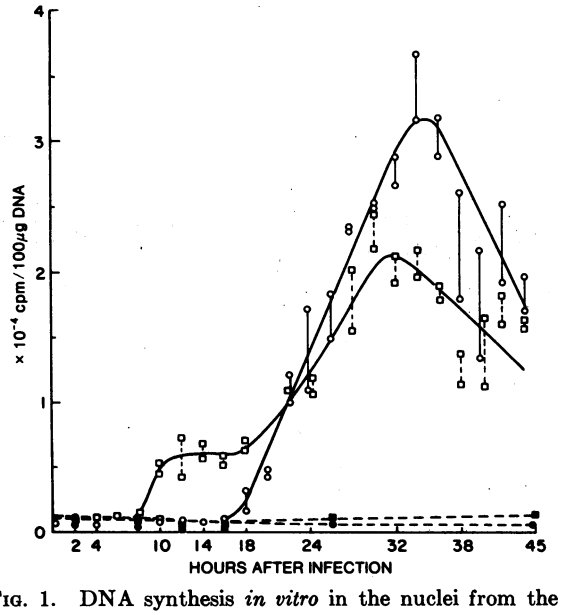

FIG. 1. DNA synthesis *in vitro* in the nuclei from the cells isolated various times after infection. ^{3}H radioactivity in Hirt supernatant (○——○) and Hirt pellet (□——□); ^{3}H radioactivity in Hirt supernatant (●- - -●) and Hirt pellet (■- - -■) from the nuclei of mock-infected cells. Ranges of values are shown.

Isopycnic Banding of DNA in Propidium Iodide–CsCl. To dialyzed Hirt supernatants, CsCl was added to a final density of 1.504 g. cm^{-3} and propidium iodide to a final concentration of 0.2 mg/ml. Marker SV40 [^{14}C]DNA was added and the final volume was adjusted to 4.75 ml. Gradients were centrifuged for 72 hr at 20° in a Spinco SW 65 rotor at 32,000 rpm. Fractions were collected and counted in toluene–triton X-100 scintillation fluid.

RESULTS

Synthesis of Viral and Host DNA in the Isolated Nuclei. Nuclei were isolated from the infected cells at various times after infection and incubated for 15 min with [^{3}H]dTTP, as described above. Viral and cellular DNA were separated by the Hirt procedure and analyzed for radioactivity (Fig. 1). The number of nuclei and the amount of DNA for each time point were measured and the results were expressed as cpm/-100 μg of nuclear DNA. For the first 8 hr after infection, nuclei did not incorporate any [^{3}H]dTTP into DNA. Between 10 and 18 hr after infection, nuclei incorporated detectable quantities of [^{3}H]dTTP only into the Hirt pellet, which contained only host DNA. Sixteen to 18 hr after infection, nuclei synthesized both host and viral DNA. A similar time sequence of host and viral DNA synthesis was observed *in vivo* (16) and was reproducible under the conditions of infection described above. Experiments in which 30-sec pulses of [^{3}H]dT were given at intervals after infection *in vivo* showed that host DNA synthesis starts between 10 and 12 hr after infection and reaches a maximum between 20 and 22 hr. Viral DNA synthesis started at 16–18 hr after infection and reached a maximum between 34 and 36 hr after infection. After viral DNA synthesis had started, the nuclei showed increased incorporation of [^{3}H]dTTP into the Hirt pellet. This increased capacity to synthesize host DNA was observed in only the *in vitro* nuclear system. There was a maximum incorporation of [^{3}H]dTTP into host DNA in the nuclei of cells 30–32 hr after infection. The maximum viral DNA synthesis occurred in nuclei 32–34 hr after infection, which was also observed in labeling experiments *in vivo*.

Viral DNA synthesized *in vitro* from nuclei 26 hr and 35 hr after infection was sedimented through neutral and alkaline sucrose gradients; the results are shown in Fig 2. Most of the radioactive DNA sedimented in a neutral gradient as a broad peak (about 22 S), ahead of marker SV40 component I, and some sedimented at a position of SV40 component II. A portion of the radioactive DNA sedimented at 5–7 S. Although the proportion of this species of DNA appeared to be considerably less at 26 hr than at 35 hr, only one experiment was carried out at 26 hr, whereas in several experiments at 35 hr the proportion varied. About 50% of the radioactivity sedimented in the 3–7S region in an alkaline gradient and the rest in the 7–16S region. When samples were sedimented in alkaline sucrose gradients under conditions in which a 53S peak of

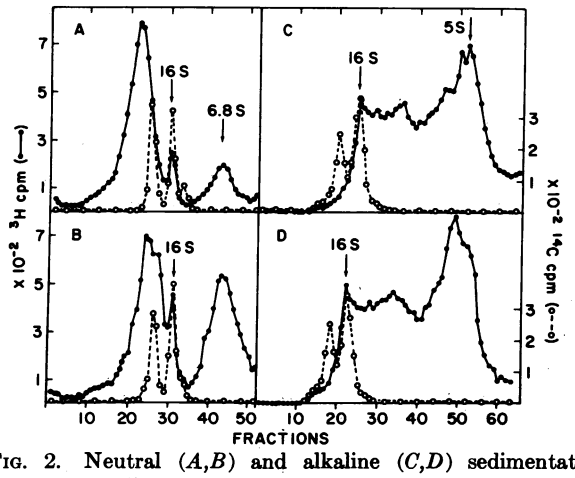

FIG. 2. Neutral (*A,B*) and alkaline (*C,D*) sedimentation analysis of 15-min DNA synthesized *in vitro* (Hirt supernatant) in the nuclei from cells 26 hr (*A,C*) and 35 hr (*B,D*) after infection. Sedimentation is from right to left.

supercoiled SV40 DNA (component I) could be observed, no radioactivity was detected in this region.

Dialyzed Hirt supernatant was banded in a CsCl–propidium iodide gradient (Fig. 3). About 60% of the DNA banded at the density of marker SV40 component II and about 40% banded as replicative intermediate (15) between marker SV40 component I and component II. The synthesized DNA in the Hirt supernatant was hybridized with unlabeled SV40 and BSC-1 DNA on filters in 50% formamide at 37° (17). The [^{3}H]DNA hybridized specifically with SV40 DNA (Table 1).

Kinetics of DNA Synthesis in Isolated Nuclei. DNA synthesis continues in the isolated nuclei for only 4–5 min (Fig. 4). The rate of viral DNA synthesis (supernatant) is linear for the first 3 min and then ceases abruptly within 4–5 min. Host DNA (Hirt pellet) is synthesized at a slower rate than viral DNA and also decreases at 4–5 min, but the cessation is less abrupt.

TABLE 1. *Hybridization of DNA, synthesized in vitro, in Hirt supernatant with SV40 and BSC-1 DNA on filters*

	Input counts	% of Input counts bound: SV40 DNA 10 μg per filter	% of Input counts bound: BSC-1 DNA 35 μg per filter
SV40 [^{3}H]DNA	1940	87.4	—
Vero [^{3}H]DNA	2640	—	16.1
[^{3}H]DNA synthesized *in vitro*	1085	73.5	3.0

Hybridization was done at 37° for 24 hr in 50% formamide, 0.5% sodium dodecyl sulfate, 0.75 M NaCl, and 0.05 M Tris·HCl (pH 7.3). SV40 [^{3}H]DNA, 12,900 cpm/μg: Vero [^{3}H]DNA, 19,410 cpm/μg. DNA filters were obtained from Dr. K. Rao.

Hirt supernatants from 30-sec and 5-min incubations were analyzed on neutral and alkaline sucrose gradients (Fig. 5). About 45% of the radioactive DNA from the 30-sec sample sedimented as a broad peak in the 5–7S region in a neutral sucrose gradient and the rest as a sharp peak at the marker, SV40 component I position. The 5-min sample showed a broader radioactive DNA peak at the component-I region but contained about the same proportion of DNA in the 5–7S region as in the 30-sec sample. On alkaline gradient analysis, about 50% of the DNA from the 30-sec sample sedimented as 3–7S DNA fragments. After 5 min of synthesis, about 45% of the DNA again sedimented at the 3–7S region except that the peak was broader than that of the 30-sec sample.

Pulse-Chase Experiments. Nuclei were incubated in the presence of [^{3}H]dTTP for 45 sec and then radioactivity was chased with unlabeled dTTP. Hirt supernatants from samples chased after 0 min and 15 min were analyzed on neutral and alkaline sucrose gradients (Fig. 6). Radioactivity from the 5 to 7S peak of the neutral gradient was not chased into the component-I region, nor was the 3–7S peak chased into the 7–16S region, as analyzed in alkaline gradients. These peaks only be-

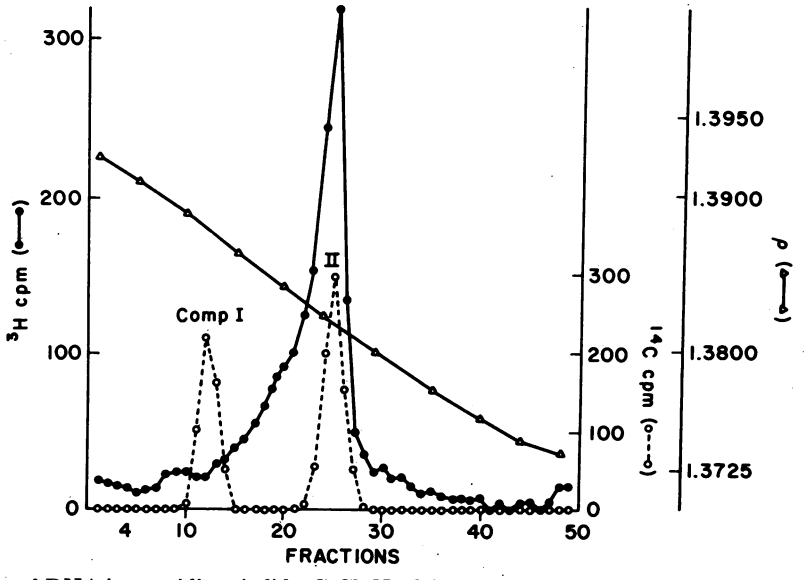

FIG. 3. Isopycnic banding of DNA in propidium iodide–CsCl. Nuclei from cells 35 hr after infection were incubated with [^{3}H]dTTP. An aliquot of dialyzed Hirt supernatant was centrifuged to equilibrium as described in *Methods*.

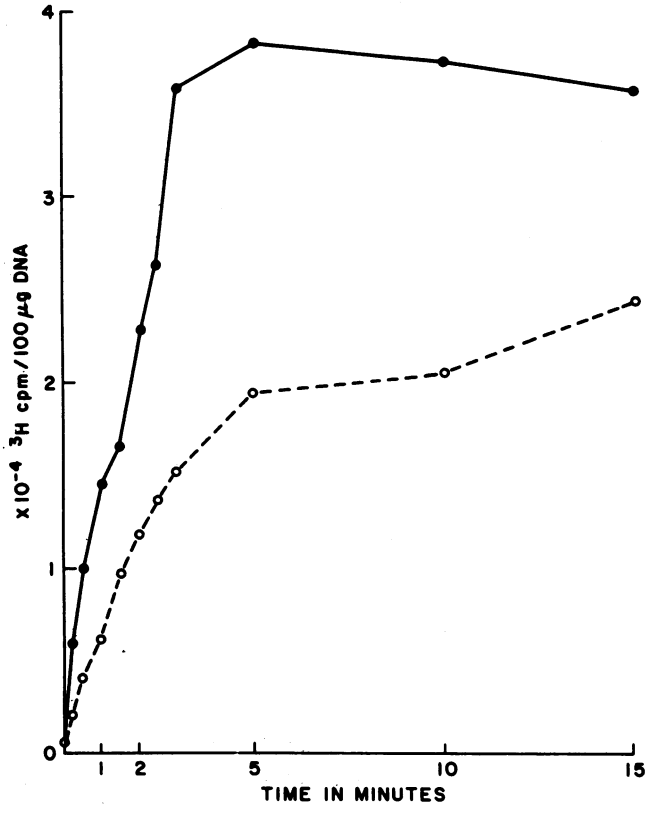

FIG. 4. Kinetics of DNA synthesis *in vitro* in the nuclei from cells 35 hr after infection. Hirt supernatant (O——O); Hirt pellet (O- - -O).

come broader after the chase period. The proportions of 3–7S peak remained about 50%, both before and after the chase period. When the concentration of dTTP during the chase period was twice that in Fig. 6, no difference was seen.

DISCUSSION

Nuclei isolated from AGMK cells infected with SV40 synthesized DNA *in vitro* when they had the capacity to do so in the intact cell. These nuclei, isolated at different times after infection, synthesized either only host or both host and vira DNA *in vitro*, retaining the same temporal relationships as those seen in the intact cell. Nuclei isolated any time between 10 and 18 hr after infection incorporate the same amounts of [³H]dTTP into the host DNA. Late in infection, nuclei that synthesize maximum viral DNA *in vitro* also incorporate maximum [³H]dTTP into the host DNA. This anomaly, not observed *in vivo*, needs further investigation.

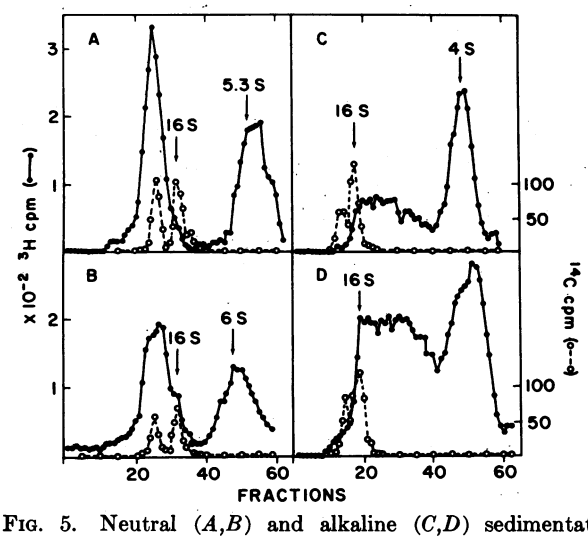

FIG. 5. Neutral (*A,B*) and alkaline (*C,D*) sedimentation analysis of DNA synthesized *in vitro* (Hirt supernatant) in the nuclei from cells 35 hr after infection (*A,C*) 30-sec and (*B,D*) 5-min incubations.

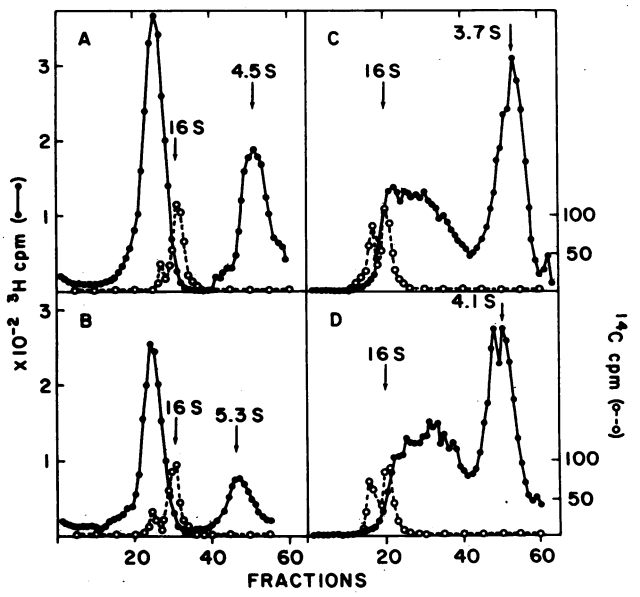

FIG. 6. Pulse-chase experiment. Nuclei from cells 34 hr after infection were incubated for 45 sec in the presence of 1 μM [³H]dTTP (30,000 cpm/pmol) as described in *Methods*. Radioactivity was chased by addition of 0.1 mM unlabeled dTTP and incubation was continued further for 15 min. Aliquots of Hirt supernatants were centrifuged in neutral (*A,B*) and alkaline (*C,D*) sucrose gradients. Synthesis (45-sec): 0-min chase (*A,C*) and 15-min chase (*B,D*). The total amount of ³H counts in Hirt supernatant was 16,075 cpm at 0-min, 15,600 cpm at 5-min, and 18,760 at 15-min chase periods.

The results of viral DNA synthesis *in vitro* reported here are consistent with the discontinuous mechanism of SV40 DNA replication *in vivo* (8). Incorporation of [³H]dTTP into isolated nuclei is linear for 3–4 min and then stops abruptly. It is incorporated simultaneously and in nearly equal proportion into both 3–7S and 7–16S DNA, as detected in alkaline gradients. *In vivo* 3–7S fragments labeled in 30 sec with [³H]dT have been observed to be chased into 7–16S DNA (7), and this finding was confirmed in our laboratory. However, attempts to chase 3–7S fragments into 7–16S DNA failed in our isolated nuclear system. During polyoma DNA replication RNA primer has been found to be covalently attached to 4S fragments of DNA (10). Our preliminary experiments indicate that with SV40 DNA synthesized *in vitro*, RNA primer was also associated with a replicative intermediate (results to be published). The involvement of the following steps during DNA replication have been proposed (10, 11). (*1*) Synthesis of RNA primer, (*2*) synthesis of 4S DNA on RNA primer, (*3*) removal of primer ribonucleotides by a specific RNase, (*4*) filling in the gaps between the fragments with deoxynucleotides, and (*5*) joining of fragments with ligase. With these steps in mind, as well as the results presented here, I would like to present the following model for replication at the replicating fork.

In the "replicating complex," at the replicating fork parental DNA strands are separated and RNA primer is synthesized on both strands. Then 3–7S DNA is synthesized on each strand, using the RNA primer. Thereafter, RNA primer is removed and the process is repeated as the replicating fork moves ahead. The gaps between the replicating fragments are then filled in by a DNA polymerase and joined by a ligase. The simultaneous incorporation of [^{3}H]dTTP into 3–7S DNA fragments and 7–16S DNA in our system supports the idea that the filling of gaps is independent of the synthesis of 3–7S fragments. Since 3–7S fragments are not chaseable in this *in vitro* nuclear system, the removal of RNA or the elongation of the replicating fork may be impaired. These processes may require factors that are lost or inactivated during the isolation of nuclei. The linear kinetics of incorporation of [^{3}H]dTTP for 3–4 min, followed by the abrupt cessation of DNA synthesis, is consistent with the idea of defective maturation. Thus, DNA synthesis occurs only at the sites where 3–7S fragments can be made and where the filling-in process can take place. During treatment of infected cells with hydroxyurea or 5-fluordeoxyuridine, only 3–7S fragments are synthesized (10–12). These data suggest that the drugs affect only the filling-in process, whereas they do not block synthesis of 3–7S fragments. These newly synthesized fragments of DNA, in the absence of the maturation process, tend to dissociate from parental strands both in this *in vitro* nuclear system and *in vivo* during 5-fluorodeoxyuridine or hydroxyurea treatment. The easily dissociable DNA fragments are then detectable as 5–7S DNA on neutral gradients.

The simultaneous incorporation and nearly equal distribution of [^{3}H]dTTP between 3–7S and 7–16S DNA can also be explained on the basis of the possibility that DNA synthesis is continuous on one strand and discontinuous on another strand. However, I would tend to discard this possibility since Fareed *et al.* (8) have shown that 70–90% of 4S fragments made *in vivo* self-anneal.

In the nuclear system described here, some of the individual steps involved during maturation of DNA fragments are observable, thereby allowing the further study of the process of maturation. Preliminary experiments indicate that the maturation can proceed in a similar *in vitro* system, but under conditions different from those described here.

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