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RNA-Linked Short DNA Fragments During Polyoma Replication

(isolated nuclei/discontinuous synthesis/deoxynucleotide starvation/hydroxyurea)

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ABSTRACT **During *in vitro* incubation, nuclei from polyoma-infected cells elongate the daughter strands of the replicative intermediate of polyoma DNA. This process is now shown to involve the transient formation of short fragments (4–5 S), a process that is stimulated by the addition of ribonucleoside triphosphates. The presence of stretches of RNA at the 5′-end of short DNA chains was determined from Cs_2SO_4 equilibrium centrifugation and from the finding that isotope from α-^{32}P-labeled deoxynucleoside triphosphates was recovered in 2′(3′)-ribonucleotides after alkaline hydrolysis. Transfer occurred preferentially with [α-^{32}P]dCTP as substrate. Starvation for deoxynucleotides by *in vivo* treatment with hydroxyurea resulted in the accumulation of short fragments that are deficient in RNA. Our results suggest that a late step during the discontinuous synthesis of polyoma DNA is selectively inhibited when deoxynucleotides are in short supply.**

Isolated nuclei from polyoma-infected 3T6 (mouse fibroblast) cells continue to perform a limited replication of viral DNA *in vitro* (1–3). Only the replicative intermediate of polyoma DNA, present in the cells at the time of isolation of the nuclei, participates during the *in vitro* reaction, which represents an elongation of daughter strands by semiconservative replication (3).

Recent work with microbial systems by Kornberg and coworkers (4, 5) and Sugino *et al.* (6) has implicated RNA as a primer of DNA replication. We will present evidence for a similar participation of RNA during polyoma DNA replication in isolated nuclei. This process appears to involve the intermediate formation of short fragments, which sediment at about 4–5 S, consisting of mixed RNA–DNA polynucleotides. Short chains also accumulate *in vivo* during deoxynucleotide starvation after addition of hydroxyurea.

MATERIALS AND METHODS

Labeled nucleoside triphosphates were purchased from New England Nuclear Corp. or were synthesized by the procedure of Symons (7). Other sources of materials, the infection of cells, the preparation of polyoma DNA, and the centrifugation procedures were as described (1–3). Nuclei were prepared from cells suspended in "isotonic Hepes" buffer (2) by Perry's procedure with 0.5% NP-42 (8), rather than by homogenization in "hypotonic Hepes" buffer (2). After centrifugation, the nuclei were resuspended in 1–4 volumes of isotonic buffer and incubated under standard conditions (2). In some experiments ribonucleoside triphosphates were added, as indicated in the legends.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid.

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RESULTS

Intermediate formation of short fragments

Infected nuclei were incubated under standard conditions with [^{3}H]dTTP, and the radioactive polyoma DNA formed was analyzed by centrifugation in alkaline sucrose gradients (Fig. 1). At short incubation times, radioactivity was distributed bimodally, with a sharp peak around 4–5 S and a second, broader, peak at higher S-values. The relative amount of radioactivity present in this second peak increased with time. The different labeled species were shown to be polyoma specific by hybridization studies.

In a pulse–chase experiment, the radioactivity from the short chains formed during the first 1.5 min was converted into longer chains on prolonged incubation (Fig. 2). These results suggest that the DNA sedimenting at 4–5 S represents an intermediate in the synthesis of longer chains.

Involvement of RNA in the synthesis of short fragments

ATP enhances the incorporation of [^{3}H]dTTP into polyoma DNA (2). Addition of a mixture of CTP, GTP, and UTP further stimulated the reaction slightly. Alkaline sucrose gradient centrifugation revealed that ribonucleoside triphosphates specifically increased the formation of short chains after 5 min of incubation (Fig. 3). This stimulation was highly reproducible with different preparations of nuclei, and was identical at both standard (2 mM) and suboptimal (0.2 mM) ATP concentrations. The results suggest that ribonucleotides are involved in the formation of the short fragments, possibly in their initiation, though ATP undoubtly serves other roles as well. The effect of ribonucleoside triphosphates during a 1.5-min incubation was very small (Fig. 3). This finding could be explained if most of the DNA synthesis represented elongation of chains already initiated *in vivo*, or if RNA synthesis was performed with remaining pools of ribonucleoside triphosphates as precursors.

The 4–5S fragments thus appear to contain stretches of RNA similar to the "Okazaki-fragments" formed during DNA replication in *Escherichia coli* (6, 10). In order to demonstrate the association of RNA with DNA, we performed equilibrium centrifugation studies in neutral Cs_2SO_4 (10). Infected nuclei were incubated for 1.5 and 5 min with [^{3}H]dTTP under

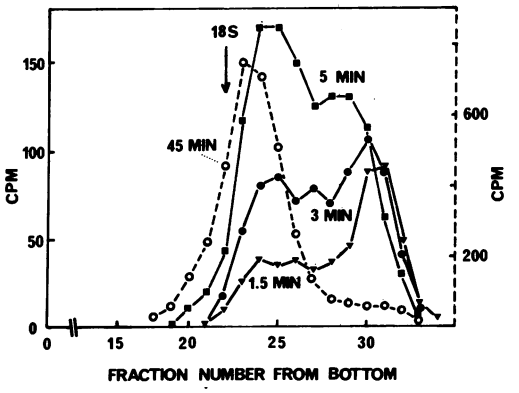

FIG. 1. Alkaline sucrose gradients of polyoma DNA synthesized for different times. Nuclei were incubated at 25° with [^{3}H]dTTP (2,300 cpm/pmol). Hirt supernatants (9) were prepared (2), and identical aliquots were centrifuged together with marker polyoma [^{14}C]DNA through an alkaline sucrose gradient (2) for 2.5 hr at 55,000 rpm and 4° in a Spinco SW56 rotor. Fractions were analyzed for acid-insoluble counts.

standard conditions. The isolated polyoma replicative intermediates were denatured at 100° and centrifuged to equilibrium, together with marker polyoma [^{14}C]DNA. Heat-denatured DNA obtained from either the 1.5- or 5-min incubation banded at densities higher than the DNA marker, and such a shift did not occur after treatment with alkali (Fig. 4). This result indicates a linkage of the polyoma DNA to RNA.

The higher banding densities of the sample incubated for 1.5 min implies that the RNA–DNA ratios of the mixed polynucleotides are greater at the shorter incubation time. This result could be due either to elongation of the DNA chains or to removal of RNA.

Further evidence for a RNA–DNA species was obtained from similar experiments with [^{3}H]UTP and [^{3}H]CTP used to label RNA, together with [^{14}C]dATP to label DNA. Equilibrium centrifugation (Fig. 5) showed a large tritium peak at a density of 1.66 g/ml (= RNA), and a second, much smaller peak at a density corresponding to that of [^{14}C]DNA (1.49 g/ml). Thus, RNA linked to polyoma DNA was synthesized during the *in vitro* incubation. The relative sizes

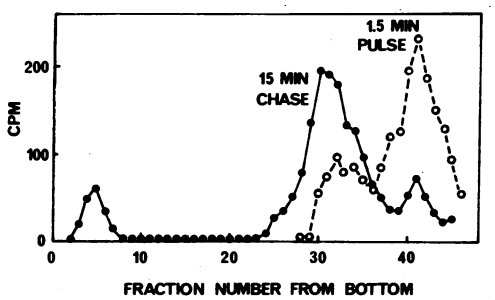

FIG. 2. Effect of incubation (chase) with unlabeled precursor on 4–5S fragments. Nuclei were first incubated for 1.5 min at 25° with [^{3}H]dTTP (7000 cpm/pmol). A 50-fold excess of unlabeled dTTP was added, and incubation was continued for 15 min at 37°. Aliquots of Hirt supernatants were analyzed by alkaline sucrose centrifugation as described in Fig. 1. The total amount of radioactivity in the Hirt supernatants was 7400 cpm before and 8600 cpm after the chase.

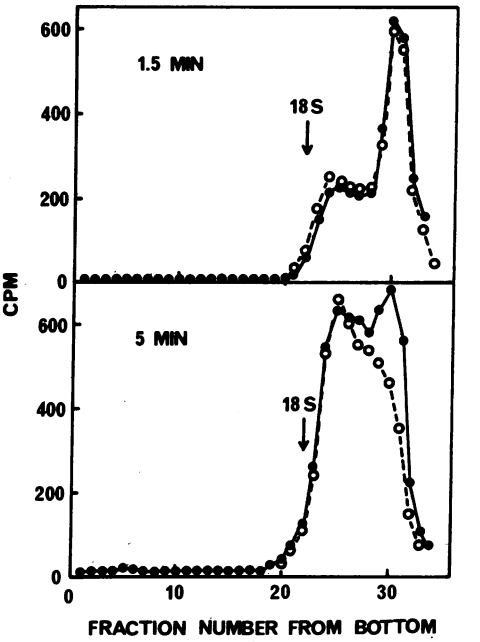

FIG. 3. Effect of ribonucleoside triphosphates on synthesis of 4–5S fragments. Nuclei were incubated for 1.5 or 5 min, either under standard conditions (O– – –O) or under standard conditions with 60 μM (each) CTP, GTP, and UTP added (●——●). The data were obtained from identical aliquots run in parallel on alkaline sucrose gradients. The total amount of radioactivity in the Hirt supernatants was, at 1.5 min, 18,000 (no ribonucleotides) and 19,000 (with ribonucleotides); at 5 min, 37,000 (no ribonucleotides) and 41,000 (with ribonucleotides).

of the ^{14}C and ^{3}H peaks at ρ = 1.49 show that most of the DNA-linked RNA was synthesized between 1.5 and 5 min (see also Fig. 3). After 15 min, the relative size of the ^{3}H peak associated with the DNA-peak decreased considerably. Alkaline treatment of samples before centrifugation removed all their ^{3}H label and caused banding of the ^{14}C-labeled material at the density of DNA (results not shown). These experiments suggest that RNA may be associated with nascent DNA *transiently*, with RNA being removed during elongation of the DNA chains.

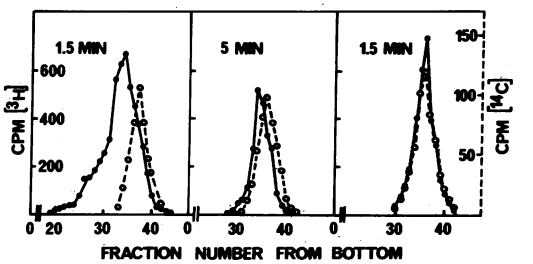

FIG. 4. Cs_2SO_4 equilibrium centrifugation of denatured, newly synthesized polyoma DNA. Nuclei were incubated for 1.5 or 5 min at 25° with [^{3}H]dTTP (7000 cpm/pmol) and 60 μM (each) CTP, GTP, and UTP. Polyoma replicative intermediate was purified by phenol extraction and chromatography on benzoylated, naphthoylated DEAE–cellulose (2), denatured, and centrifuged to equilibrium in neutral Cs_2SO_4 (6) for 48 hr at 36,000 rpm and 15° in a SW50 rotor. Sonicated polyoma [^{14}C]-DNA was added as a marker before denaturation. The samples were heated for 5 min at 100° in either 30 mM NaCl–3 mM Na citrate (*left & center*), or 0.3 M NaOH (*right*).

TABLE 1. *Formation of a covalent RNA–DNA link during polyoma DNA synthesis. Transfer of isotope from [α-^{32}P]-deoxynucleoside triphosphates to RNA*

α-^{32}P substrate	% Acid-soluble ^{32}P after alkali	% ^{32}P in			
		Ap	Cp	Gp	Up
dATP	0.4	55	14	17	13
dCTP	2.0	19	30	26	26
dGTP	1.1	26	9	37	28
dTTP	0.3	32	15	18	35

Nuclei (1.0–1.5 mg of DNA) were incubated for 5 min at 25° in a final volume of 1.2 ml under standard conditions with 60 μM each of CTP, GTP, and UTP, and with either of α-^{32}P-labeled dATP, dCTP, dGTP, or dTTP (15,000–25,000 cpm/pmol). Cs_2SO_4 centrifugation (see Fig. 5) of the Hirt supernatants revealed that all incorporation was into DNA. After passage through a column of Biogel P-30, the material (0.5 to 1.5 × 10^6 cpm) was precipitated with $HClO_4$, washed, and digested with 0.3 M KOH for 16–20 hr at 37°. The supernatant solution, after precipitation with $HClO_4$, was neutralized with KOH and concentrated under reduced pressure. Aliquots of this solution were chromatographed on Whatman 3 MM paper in solvents A and C of Flügel and Wells (12). ^{32}P was found only at the origin and in the areas corresponding to the four ribonucleotides. Computations of base ratios do not include material at the origin, which did not exceed 6% (except with dGTP as substrate). With dGTP, up to 35% of the radioactivity was found close to the origin, and a corresponding correction was made for the calculation of the % acid-soluble ^{32}P after alkali.

Proof for a covalent linkage of RNA to nascent DNA was obtained from transfer from [α-^{32}P]-labeled deoxynucleoside triphosphates into 2′(3′)-ribonucleotides after alkaline hydrolysis of the mixed polynucleotides (5, 11, 12). The products of separate incubations with ^{32}P-labeled nucleotides were analyzed by paper chromatography (Table 1). All four deoxynucleotides and ribonucleotides were found at the RNA–DNA link. However, dCMP was the preferred deoxynucleotide, and started about 50% of the DNA chains. There was no clear preference for the terminating ribonucleotide.

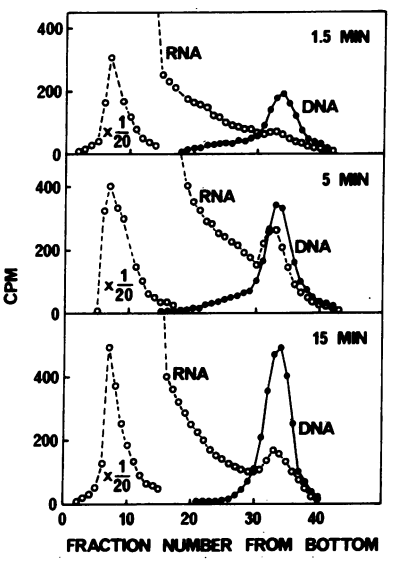

FIG. 5. Cs_2SO_4 equilibrium centrifugation of denatured DNA synthesized from [^{14}C]dATP and [^{3}H]UTP + [^{3}H]CTP. Nuclei were incubated at 25° for 1.5, 5, or 15 min with [^{14}C]dATP (900 cpm/pmol), 60 μM GTP, and 10 μM (15,000 cpm/pmol) [each] [^{3}H]CTP and [^{3}H]UTP. Polyoma replicative intermediate was purified and centrifuged as described in Fig. 4. The samples were heated in 30 mM NaCl–3 mM Na citrate for 5 min at 100° before centrifugation. Note the change in scale from fractions below 15.

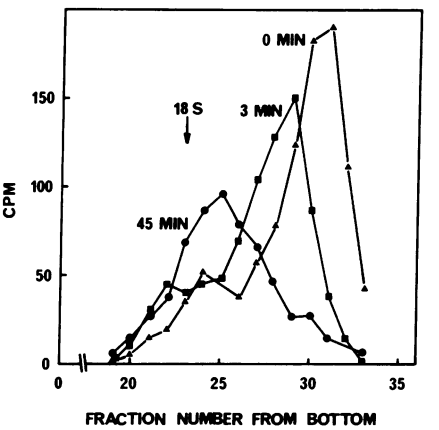

FIG. 6. *In vitro* chase of fragments formed during deoxynucleotide starvation *in vivo*. 3T6 cells (28 hr after infection) were treated for 10 min with 10 mM hydroxyurea, then pulsed for 10 min with 1.5 μM [^{3}H]dT (20 Ci/mmol) in the presence of the drug. Nuclei were prepared and incubated for different time periods at 25° with unlabeled dTTP. Identical aliquots of Hirt supernatants were centrifuged on alkaline sucrose gradients as described in Fig. 1. The total amount of ^{3}H in the Hirt supernatants was 7200 cpm at 0 min, 7400 cpm at 3 min, and 6400 cpm at 45 min.

Short fragments accumulate *in vivo* during deoxynucleotide starvation

Treatment of cells in tissue culture with hydroxyurea rapidly depletes the dGTP and dATP pools (13). Addition of 10 mM hydroxyurea to polyoma-infected cells reduced the incorporation of [^{3}H]thymidine (dT) into viral DNA to about 10% within a few minutes (Magnusson and Reichard, to be published). The newly synthesized DNA was polyoma-specific, and consisted mainly of short fragments sedimenting under both alkaline and neutral conditions at about 4 S. These fragments were single-stranded, as they were digested by exonuclease I (14) but not by exonuclease III (15). Similar single-stranded DNA was earlier observed after pulse labeling of *E. coli* (16, 17) and *Bacillus subtilis* (17). Furthermore, addition of hydroxyurea to cells containing labeled replicative intermediate or Form I of polyoma DNA did not cause formation of 4S fragments. As a consequence, these fragments are not breakdown products of polyoma DNA.

By the following pulse–chase experiment, we could show that the short fragments were precursors of long chains. Cells were labeled *in vivo* with [^{3}H]dT in the presence of hydroxyurea, and the isolated nuclei were incubated for different times with unlabeled dTTP. Under alkaline conditions, 80% of the *in vivo* labeled DNA sedimented at about 4 S (Fig. 6). During the *in vitro* chase, the label was transferred progressively into heavier chains. Similar centrifugation studies at neutral pH (not shown) demonstrated that the 4S material was transferred to the position of the replicative

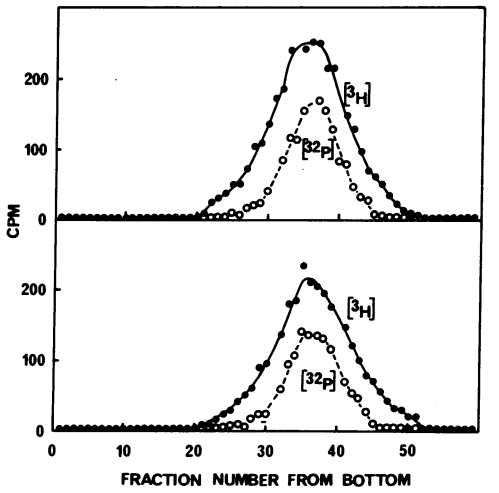

FIG. 7. Cs_2SO_4 equilibrium centrifugation of denatured fragments formed during deoxynucleotide starvation. Infected cells were labeled with [^{3}H]dT as described in Fig. 6. DNA was extracted by the Hirt procedure, purified by benzoylated, naphthoylated DEAE–cellulose chromatography, and centrifuged to equilibrium in neutral Cs_2SO_4 after heating for 10 min at 100° in either 0.15 M NaCl–15 mM Na citrate (*top*) or 0.3 M KOH (*bottom*). Sonicated [^{32}P]DNA from *E. coli* was added as a marker before the heating step.

intermediate (25 S). Both the 4S and 25S DNA were shown to be polyoma specific by hybridization studies.

Does the 4S species formed during deoxynucleotide starvation represent accumulation of an intermediate *identical* to the short fragments formed during polyoma DNA replication *in vitro*? Since the fragments formed *in vitro* contained stretches of RNA, we analyzed the 4S species formed during hydroxyurea treatment by Cs_2SO_4 equilibrium centrifugation. The small, though significant, displacement of the ^{3}H-labeled sample relative to the [^{32}P]DNA marker (compare Fig. 7, *top* and *bottom*) suggests that the short fragments formed in the presence of hydroxyurea contain considerably less RNA than the amount associated with the fragments formed during short incubation times *in vitro* (Fig. 4). The greater band width for the hydroxyurea fragments, as compared to the material labeled for short periods *in vitro* (compare alkali-treated controls Fig. 7, *bottom* and 4, *right*), is consistent with the smaller average chain length of the hydroxyurea fragments.

DISCUSSION

From our data we would like to suggest the following two points concerning the replication of polyoma DNA: (*i*) short fragments that sediment at 4–5 S are formed early during replication; therefore, at least in part, replication proceeds discontinuously. A similar conclusion was recently reached for the *in vivo* replication of SV40 DNA (18). Our results do not reveal whether only one or both daughter strands are synthesized in this manner. (*ii*) These fragments are initiated by RNA linked covalently to the 5′-terminus of the DNA chain, and dCMP was the preferred deoxynucleotide at the RNA–DNA link. The replication of polyoma DNA thus shows a considerable similarity to the replication of *E. coli* DNA, as outlined by the work of Okazaki and coworkers (6, 10), although the short fragments involved in polyoma replication appear to be of a smaller size than the "Okazaki-fragments."

Accumulation of short fragments during deoxynucleotide starvation is of particular interest, since these species may represent intermediates formed in the course of normal DNA synthesis. Although the origin of these fragments and their single-stranded nature after isolation at neutral pH is not completely clear, the fragments are polyoma-specific and behave kinetically as precursors of polyoma replicative intermediate. Their accumulation presumably results from inhibition of a step involved in the discontinuous synthesis of polyoma DNA. Schematically this process can be visualized to proceed in five steps: (*i*) initiation by ribonucleotides; (*ii*) chain elongation by deoxynucleotides; (*iii*) removal of initiating ribonucleotides; (*iv*) gap-filling by deoxynucleotides; and (*v*) joining of fragments. It seems conceivable that steps (*ii*) and (*iv*) are catalyzed by two different polymerases, and that the enzyme catalyzing the latter step is preferentially blocked when deoxyribonucleotides are in short supply. Alternatively, either step (*iv*) or (*v*) may be subject to allosteric regulation by deoxynucleotides.

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1. Winnacker, E. L., Magnusson, G. & Reichard, P. (1971) *Biochem. Biophys. Res. Commun.* **44**, 952–957.
2. Winnacker, E. L., Magnusson, G. & Reichard, P. (1972) *J. Mol. Biol.*, in press.
3. Magnusson, G., Winnacker, E. L., Eliasson, R. & Reichard, P. (1972) *J. Mol. Biol.*, in press.
4. Brutlag, D., Schekman, R. & Kornberg, A. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2826–2829.
5. Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L. L. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2691–2695.
6. Sugino, A., Hirose, S. & Okazaki, R. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1863–1867.
7. Symons, R. H. (1969) *Biochim. Biophys. Acta* **190**, 548–550.
8. Perry, R. P. & Kelly, D. E. (1968) *J. Mol. Biol.* **35**, 37–59.
9. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369.
10. Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., Kainuma, R., Sugino, A. & Iwatsuki, N. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33**, 129–142.
11. Keller, W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1560–1564.
12. Flügel, R. M. & Wells, R. D. (1972) *Virology* **48**, 394–401.
13. Skoog, L. & Nordenskjöld, B. (1971) *Eur. J. Biochem.* **19**, 81–89.
14. Lehman, I. R. & Nussbaum, A. L. (1964) *J. Biol. Chem.* **239**, 2628–2636.
15. Richardson, C. C., Lehman, I. R. & Kornberg, A. (1964) *J. Biol. Chem.*, **239**, 251–258.
16. Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K. & Sugino, A. (1968) *Proc. Nat. Acad. Sci. USA* **59**, 598–605.
17. Oishi, M. (1968) *Proc. Nat. Acad. Sci. USA* **60**, 329–336.
18. Fareed, G. C. & Salzman, N. P. (1972) *Nature New Biol.* **238**, 274–277.