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INCORPORATION OF URACIL INTO THE GROWING STRAND OF ADENOVIRUS 12 DNA

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SUMMARY

The amount of rapidly labeled short DNA chains in adenovirus 12(Ad12)-infected cells was markedly increased in the presence of either uridine or deoxycytidine which could be converted to dUTP. When the infected cells were labeled with [^{3}H]uridine or [^{3}H] deoxycytidine and the labeled nucleotides in the short DNA chains from the Hirt supernatant were analysed by thin-layer chromatography, approximately 90 or 20% of the label was detected in dUTP. These results suggest that at least a portion of short DNA chains formed during Ad12 DNA replication is derived from an excision-repair mechanism of uracil containing nascent strands.

INTRODUCTION

Okazaki *et al.* (1) showed that a DNA chain is newly synthesized in a form of small fragments and proposed a discontinuous model of DNA replication. The small replicating intermediates are called Okazaki pieces and are present at the replication fork in a multiple variety of biological systems (2).

Although the presence of small DNA intermediates in DNA replication can be established, there is a possibility that some fractions of Okazaki pieces in *E. coli* may result from an excision-repair mechanism instead of *de novo* initiation (3). Similar phenomena were also reported in other systems (4,5,6,7,21). Furthermore, a semidiscontinuous mode of DNA replication was shown in *E. coli* (8), SV40 (9), and polyoma (10). The elongation of the DNA strand in the 3'$\longrightarrow$5' direction proceeds discontinuously while

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the elongation in the 5'→3'direction proceeds continuously.

The replication of adenovirus DNA is initiated at either 3'-end of the strand and proceeds in the 5'→ 3' direction displacing the opposite parental strand. After completion of the elongation of the daughter strand, the elongation of the other daughter strand initiates at the 3'-end of the displaced strand and proceeds again in the 5'→ 3' direction (11,12,13,14,15). According to this displacement model, the DNA synthesis does not necessarily reqire the formation of Okazaki-type fragments. However, short pieces of DNA have been observed in DNA replication of Adenovirus type 2 Ad2) (16), Ad5 (17), Ad31 (18), and CELO virus, avian adenovirus (19). However, the presence of these short pieces can not always be evidence for discontinuous DNA replication (3).

In this study, we observed the incorporation of uracil into the growing adenovirus DNA strand and the presence of uracil in the short DNA pieces pulse-labeled with [^{3}H]uridine or [^{3}H]deoxycytidine. These results suggest that a portion of Okazaki-type fragments is produced by excision-repair or other postreplication processing mechanism.

MATERIALS AND METHODS

Cell and virus. Monolayer cultures of KB cells and the wild type adenovirus type 12 (Ad12) (Huie strain) were used.

Radioactive labeling of cells. KB cells (5 x 10^5 cells/ml) were infected with Ad12 at input multiplicity of 20 PFU/cell at 37°. At 30 hr postinfection (p.i.) the cells were labeled with [^{3}H] thymidine (23.0 Ci/m mole), [^{3}H]deoxycytidine (20.0 Ci/m mole), or [^{3}H]uridine (40.0 Ci/m mole) at 100 μCi/ml for the times indicated in the text. The viral DNA was extracted by the Hirt procedure (20).

Alkaline sucrose gradients. The viral DNA in the Hirt supernatant was loaded onto 38 ml of 5 to 20% alkaline sucrose gradients in 0.3N NaOH, 0.5M NaCl, and 10 mM EDTA, and centrifuged in an SW27 rotor at 23,000 rpm for 20 hr at 4°C. The acid insoluble radioactivity in each fraction was counted.

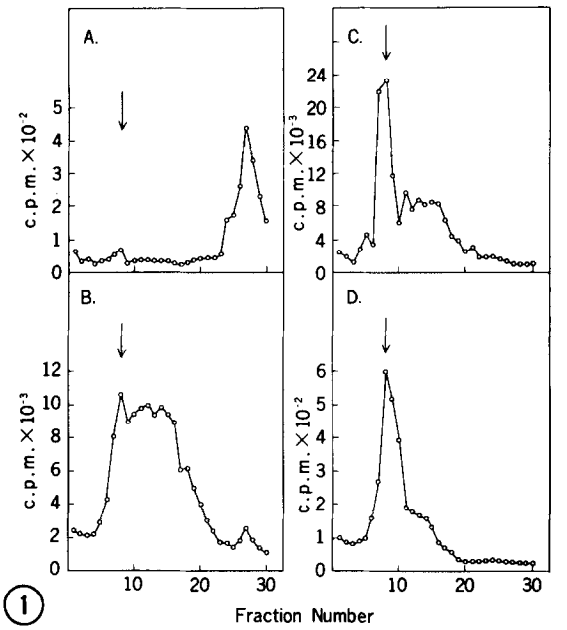
Nucleotide analysis of DNA. Either [^{3}H]uridine or [^{3}H]deoxycytidine labeled DNA in the Hirt supernatant was loaded onto a Sephadex G25 (Farmacia Fine Chemicals, Inc. "Medium" type) column (1.0 x 33 cm) equilibrated with 0.1 M NaCl, 10 mM Tris-HCl (pH 8.1), 1 mM EDTA and eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions in the void volume were collected and centrifuged in alkaline sucrose gradients as described avove. The fractions containing DNA were neutralized and extracted with phenol saturated with 10 mM Tris-HCl (pH 8.1). DNA was precipitated with ethanol. The precipitates were dissolved in buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mg/ml salmon sperm DNA) and digested in 100 μg/ml of pancreatic DNase I at 37° for 90 min. The mixture then received snake venom phosphodiesterase (final 20 μg/ml) and incubated at 37° for 30 min. UMP and dUMP were added to the mixture. Mononucleotides in the mixture were separated by two-dimensional thin-layer chromatography on 20 x 20 cm PEI-cellulose plate (polygram, Germany). The first-dimension solvent was 0.5M lithium formate buffer (pH 3.5). The second-dimension solvent was 0.2M LiCl, 20 mM Tris-HCl (pH 8.0). The nucleotide spots were visualized with UV light and their radioactivities were counted.

RESULTS AND DISCUSSION

Sedimentation analysis of Ad12 DNA pulse labeled with [^{3}H]thymidine.

KB cells were infected with Ad12 and pulse labeled with [^{3}H]thymidine for 0.5, 10, and 60 min at 30 hr p.i.. The size of labeled viral DNA was analyzed by alkaline sucrose gradients (Fig. 1). A single major peak of 6 to 7S was detected at 0.5 min pulse (Fig. 1A). At 10 min pulse, a majority of the label was distributed between about 20S and 34S (the mature size of Ad12 DNA). Only a minor peak was detected at 7S (Fig. 1B). At 60 min pulse, most of the label was detected at 34S (Fig. 1C). When 0.5 min pulsed DNA was chased for 60 min in the presence of cold thymidine, the label at the 7S peak was shifted to 34S (Fig. 1D). These patterns suggest that the 7S peak consists of Okazaki pieces and the strands elongated in a discontinuous mechanism. Similar phenomenon were reported in Ad31 (18), and avian adenovirus (19).

Effect of hydroxyurea and thymidine on the size of growing Ad12 DNA strands. Hydroxyurea (HU) and thymidine are potent inhibitors of the ribonucleotide reductase (Fig. 2) and inhibit DNA synthesis



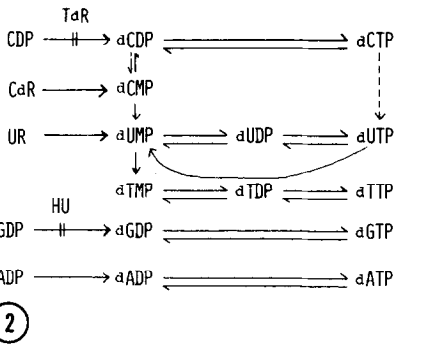


Fig. 1. Sedimentation analysis of pulse-labeled DNA of Ad12. KB cells were infected with Ad12 and labeled with [^{3}H]thymidine for the times indicated at 30 hr p.i.. A part of the pulse-labeled cells were washed twice with maintenance medium containing 0.1mM unlabeled thymidine and chased in the same medium for 60 min. Viral DNA in the Hirt supernatant was centrifuged in alkaline sucrose gradients as described in MATERIALS AND METHODS. The arrow indicates the position of ^{32}P-Ad12 DNA. A: 0.5 min pulse, B: 10 min pulse, C: 60 min pulse, D: 0.5 min pulse and 60 min chase.

Fig. 2. Pathway for deoxyribonuceotide triphosphates from precursors to DNA.

by lowering the level of intracellular pool of deoxyribonucleotide triphosphates (22) (Fig. 2). KB cells were treated with 10mM HU or 10mM thymidine for 10 min. Within 10 min, DNA synthesis was reduced to less than 5% of the control. After the treatment, the cells were labeled with [^{3}H]thymidine for 10 and 60 min in the presence of either HU (Fig. 3A, B) or thymidine (Fig. 3C, D). The labeled DNA was similarly analysed. Most of the label remained at 7S and the mature size DNA was scarcely detected. These results suggest that the amount of small species of DNA increases by lowering the level of intracellular pool of deoxyribonucleotide

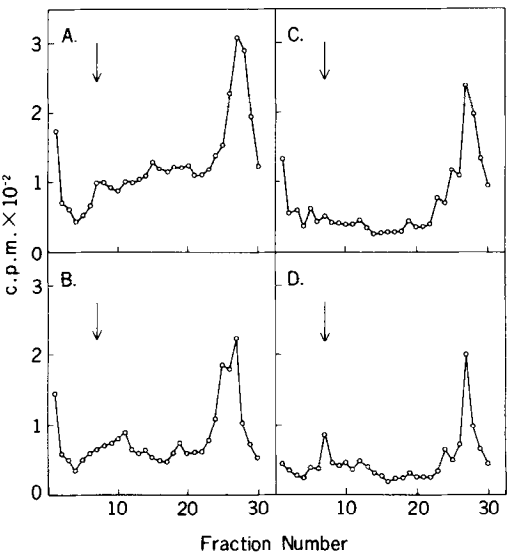


Fig. 3. Sedimentation analysis of Ad12 DNA labeled in the presence of hydroxyurea or excess thymidine.
The infected cells were pretreated with hydroxyurea (10mM) or thymidine (10mM) for 10 min at 30 hr p.i.. The cells were labeled with [^{3}H]thymidine in the presence of either of these drugs. Viral DNA was centrifuged in alkaline sucrose gradients. The arrow indicates the position of ^{32}P-Ad12 DNA. A: Treated with hydroxyurea, 10 min label. B: Treated with hydroxyurea, 60 min label. C: Treated with thymidine, 10 min label. D: Treated with thymidine, 60 min label.

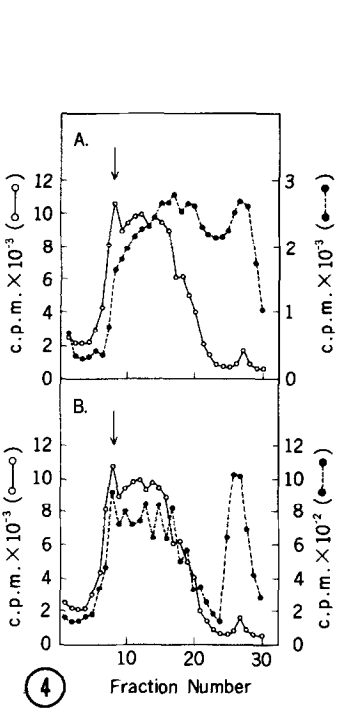
triphosphates. Similar pattern was reported in Ad5 DNA treated with HU (17). The sizes of those small DNA were the same when estimated by longer centrifugation in an SW41 rotor at 38,000 rpm for 16 hr at 4° (Data not shown). The size of this small species of DNA was reported to be larger (10-12S) in other adenovirus DNA than those presented here (6-7S) (16-19). The reason for this discrepancy has not yet been clarified.

Effect of uridine and deoxycytidine on the size of pulse-labeled Ad12 DNA. KB cells were infected with Ad12 and pulse labeled with [^{3}H]thymidine in the presence of either 10mM unlabeled uridine or deoxycytidine for 10 min at 30 hr p.i.. The radioactivity incorporated was reduced to 47 or 12% of the control by

the presence of uridine or deoxycytidine. The distribution of the label in the gradient was similar to that of the control except a marked increase in the small DNA species of about 7S (Fig. 4). These effects on the size of the pulse-labeled DNA may be due to the increased level of dUTP (Fig. 2) and a portion of small DNA species may be produced by an excision-repair of the uracil-containing labeled DNA. Similar effects were reported in E. coli (8) and polyoma DNA (6) in an in vitro experiment.

Incorporation of uracil into small DNA specics. To confirm the above suggestion, the infected cells were labeled with either [^{3}H]uridine or [^{3}H]deoxycytidine. When the cells were labeled with [^{3}H]uridine for 0.5 and 10 min, the label was present exclusively in the small DNA species (Fig. 5A, B). Longer labeling (60 min) resulted in incorporation of the label into the larger species of DNA (Fig. 5C) probably due to the conversion of [^{3}H]uridine to [^{3}H]TTP (Fig. 2). When the cells were labeled with [^{3}H]deoxycytidine for 10 and 60 min, most of the label was detected in larger species of DNA (Fig. 5E, F). The label in small DNA species, however, is greater than that appearing in cells similarly labeled with [^{3}H]thymidine (Compare Fig. 5E, F with Fig. 1B, C). This increase may reflect the conversion of a small fraction of [^{3}H]deoxycytidine to [^{3}H]dUTP (Fig. 2). The material labeled with [^{3}H]uridine was not RNA, because [^{3}H]-labeled ribosomal RNA from KB cells became acid soluble in this alkaline sucrose gradient (Data not shown).

In order to confirm further the incorporation of uracil into small DNA species, the infected cells were labeled with either [^{3}H]uridine or [^{3}H]deoxycytidine for 10 min. The Hirt supernatant from the labeled cells was loaded onto the Sephadex G25 column to



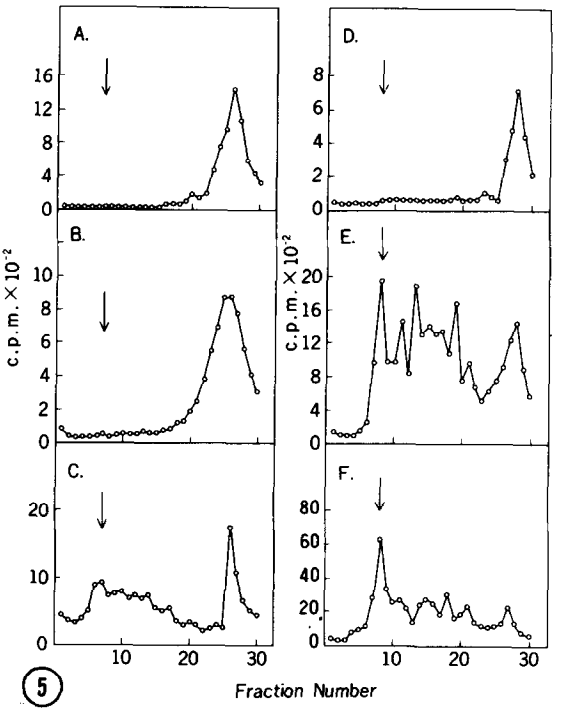


Fig. 4. Effect of uridine and deoxycytidine on the size of pulse labeled Ad12 DNA.

The infected cells were labeled with [^{3}H]thymidine in the presence of either 10mM uridine or 10mM deoxycytidine for 10 min at 30 hr p.i.. Viral DNA was centrifuged in alkaline sucrose gradients. The arrow indicates the position of ^{32}P-Ad12 DNA. A: o——o in the absence of uridine, ●----● in the presence of uridine, B: o——o in the absence of deoxycytidine, ●----● in the presence of deoxycytidine.

Fig. 5. Sedimentation analysis of Ad12 DNA from cells labeled with either [^{3}H]uridine or [^{3}H]deoxycytidine.

The infected cells were labeled with either [^{3}H]uridine or [^{3}H]deoxycytidine at 30 hr p.i.. Viral DNA was centrifuged in alkaline sucrose gradients. The arrow indicates the position of ^{32}P-Ad12 DNA. A: [^{3}H]uridine for 0.5 min, B: [^{3}H]uridine for 10 min, C: [^{3}H]uridine for 60 min, D: [^{3}H]deoxycytidine for 0.5 min, E: [^{3}H]deoxycytidine for 10 min, F: [^{3}H]deoxycytidine for 60 min.

exclude free nucleotides. Fractions in the void were pooled and centrifuged in alkaline sucrose gradients. The small DNA species was digested to 5'-mononucleotide by pancreatic DNaseI and venom phosphodiesterase. Mononucleotides were separated by PEI-cellulose thin layer chromatography and their radioactivity was determined (Table 1). dUMP, UMP, and dTMP were well separated in this

Table 1 Distribution of label in mononucleotides[a)]

Nucleotide	Uridine label		Deoxycytidine label	
	^{3}H C.P.M. (%)	^{32}P C.P.M. (%)	^{3}H C.P.M. (%)	^{32}P C.P.M. (%)
dAMP	10 (0.7)	150 (23.7)	6 (0.3)	158 (23.9)
dGMP	15 (1.1)	161 (25.4)	10 (0.6)	162 (25.2)
dCMP	20 (1.5)	149 (23.5)	1370 (79.6)	149 (23.4)
dTMP	50 (3.7)	170 (26.8)	5 (0.2)	168 (26.4)
dUMP	1250 (93.0)	3 (0.6)	330 (19.2)	5 (0.8)

a) The Hirt supernatant from the infected cells labeled with either [^{3}H]uridine or [^{3}H]deoxycytidine for 10 min was chromatographed in Sephadex G-25. Fractions in the void were pooled and centrifuged in alkaline sucrose gradients. The fraction at 6 to 7S was digested with pancreatic DNase I and venom phosphodiesterase. The digestion mixture was chromatographed with the two-dimension PEI cellulose thin layer chromatography. Nucleotide spots were cut and the radioactivity was counted.

chromatography. In [^{3}H]uridine labeled cells, most of the labeled nucleotide was dUMP and a little was dTMP. In [^{3}H]deoxycytidine labeled cells, 80% of the labeled nucleotide was dCMP and 20% of dUMP. The nucleotides derived from uniformly labeled [^{32}P]-Ad12 DNA showed an even distribution of the label in four deoxyribonucleotides. These data confirmed the conversion of [^{3}H]uridine and [^{3}H]deoxycytidine to dUTP and the incorporation of uracil into DNA.

The present data suggest that a portion of the small DNA species is formed by an excision-repair mechanism. The phenomenon might be general in many biological systems (3-7). In E. coli system, a mutation in the locus of dUTPase induced those small fragments (3, 23, 24). Mammalian cells have been known to contain a potent dUTPase activity, and a larger pool sixze of deoxyribonucleotides (22). These factors may reduce the amount of uracil incorporation into DNA. Nevertheless, a large amount of uracil is present in the small DNA species in this Ad12 system. Why the small DNA species containing many uracil rusidues is excised as a relatively unique size of 6-7S is not known.

Replication of adenovirus DNA proceeds by a displacement mechanism. The discontinuous mode of chain elongation, therefore, is not necessarily required. However, the present results are not sufficient to discriminate between the discontinuous and continuous replication of Ad12 DNA, since the size of the presumed excision fragments is similar to that of Okazaki fragments. Further studies are needed to discriminate between the alternative mechanisms.

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