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The Incorporation of Trifluorothymidine into Calf Thymus DNA in a Cell-Free System Does Not Lead to Chain Termination

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SUMMARY

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In this report we present evidence that trifluorothymidine-5′-triphosphate (F_3dTTP) once incorporated into activated calf thymus DNA by a partially purified HeLa cell cytoplasmic DNA-dependent DNA polymerase α does not lead to chain termination. Less than 1% of the [^{3}H]F_3dTTP incorporated in the cell-free system was recovered at the 3′-termini. In addition, F_3dTTP-containing DNA was capable of incorporating [^{3}H]dTTP, which led to the complete disappearance of radioactive trifluorothymidine from the 3′-termini.

Trifluorothymidine (F_3TdR) (1) is an antimetabolite of thymidine (TdR) that has been demonstrated to be a clinically highly effective drug against herpes simplex and vaccinia viral keratitis (2, 3). The drug exerts its action in relation to DNA by inhibiting thymidine kinase (4, 5), while its anabolite, trifluorothymidine 5′-monophosphate (F_3dTMP), inhibits thymidylate synthetase (6). The drug in the form of its triphosphate (F_3dTTP) retards DNA synthesis by inhibiting DNA-dependent DNA polymerases competitively with thymidine 5′-triphosphate (dTTP) and noncompetitively with other deoxyribonucleoside triphosphates (7). In addition, it is incorporated into cellular (8) and viral DNA (9), leading to the formation of DNA with a smaller sedimentation coefficient than the corresponding normal DNA (8, 9). This raised the possibility that F_3dTTP incorporation into DNA leads to chain termination, as has been reported for arabinofuranosylcytosine 5′-triphosphate (10, 11) and 3′-deoxy-3′-fluorothymidine triphosphate (12). The latter is a classical chain terminator because it lacks a hydroxyl group at the 3′-position of the deoxyribose molecule. In this communication, we present evidence that the incorporation of F_3dTTP into activated calf thymus DNA does not lead to chain termination.

HeLa cell cytoplasmic DNA polymerase α and activated calf thymus DNA were obtained as described previously (7). This DNA polymerase preparation is designated *alpha* (α) because it has a sedimentation constant of 6–8 S and requires activated DNA as the template (7). F_3dTTP and [^{3}H]F_3dTTP were synthesized and their purities checked as reported earlier (13). Nonradioactive nucleotides were obtained from Sigma Chemical Company, and [^{3}H]dTTP and calf thymus DNA were purchased from Schwarz–Mann. Protein concentration was determined by the method of Lowry *et al.* (14), and DNA polymerase activity was assayed by the filter disk method (7). One enzyme unit is defined as the incorporation of 1 pmol of [^{3}H]dTTP into acid-insoluble products per minute at 37°C.

In order to determine whether F_3dTMP in DNA would lead to chain termination, we synthesized three samples of activated calf thymus DNA using the HeLa cell DNA polymerase α: A, DNA containing [^{3}H]F_3dTMP; B, sample A elongated with [^{3}H]dTTP; and C, DNA prepared by simultaneous incubation with [^{3}H]F_3dTTP and [^{3}H]dTTP. The various DNA preparations were digested and their 3′-termini were then analyzed.

(1) DNA containing [^{3}H]F_3dTMP (Product A): The reaction mixture (1.25 ml) contained 90 nmol each of deoxyadenosine-5′-triphosphate (dATP), deoxyguanosine-5′-triphosphate (dGTP), and deoxycytidine-5′-triphosphate (dCTP), 62.5 nmol of [^{3}H]F_3dTTP (2.07 μCi), 250 μg of activated DNA, 10 μmol of $MgCl_2$, 1.25 μmol of 2-mercaptoethanol, 50 μmol of Tris–HCl buffer (pH 8.0), and 88 units of DNA polymerase α. The mixture was incubated at 37°C for 1 h, and the reaction was terminated by the addition of 500 μg of activated DNA (7) and

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12.5 μmol of EDTA and heating at 60°C for 2 min. The radioactive DNA was dialyzed against 1 liter of 50 mM Tris–HCl buffer containing 2 mmol of EDTA (pH 8.6) for 4 h and then against 1 liter of fresh buffer without EDTA overnight in a cold room.

(2) DNA containing both [^{3}H]F_3dTMP and [^{3}H]dTMP added sequentially (Product B): The reaction mixture (1.25 ml), consisting of the same components as described previously, was incubated at 37°C for 60 min. The mixture was then heated at 60°C for 2 min and dialyzed against 1 liter of 40 mM Tris–HCl buffer (pH 8.0) for 4 h and then against 1 liter of the same fresh buffer overnight in a cold room. The dialyzed DNA containing [^{3}H]-F_3dTMP was then incubated at 37°C for 30 min with 62.5 nmol of dATP, dGTP, and dCTP, 6.25 nmol of [^{3}H]dTTP (0.38 μC), 10 μmol of $MgCl_2$, 1.25 μmol of 2-mercaptoethanol, and 88 units of enzyme. The mixture was heated at 60°C for 2 min after the addition of 500 μg of activated DNA, 12.5 μmol of EDTA added, and was dialyzed extensively against 50 mM Tris–HCl (pH 8.6) containing 2 mM EDTA as described previously.

(3) DNA containing both [^{3}H]F_3dTMP and [^{3}H]dTMP added simultaneously (Product C): The reaction mixture (1.25 ml) contained 90 nmol each of dATP, dGTP, and dCTP, 31.25 nmol of [^{3}H]F_3dTTP (25 μM; 1.04 μCi), 2.5 nmol of [^{3}H]dTTP (2 μM; 3.4 μCi), 250 μg of activated DNA, 10 μmol of $MgCl_2$, 1.25 μmol of 2-mercaptoethanol, 50 μmol of Tris–HCl buffer (pH 8.0), and 88 units of enzyme. The mixture was incubated at 37°C for 60 min and, following the addition of 500 μg of activated DNA and 12.5 μmol of EDTA, was heated at 60°C for 2 min. The radioactive DNA was dialyzed against Tris–HCl buffer (pH 8.6) as described previously.

The 3′-termini of DNA were determined by digestion of the dialyzed DNA Products A, B, and C to generate 3′-deoxyribonucleotides and deoxyribonucleosides by successive treatments with micrococcal nuclease (12,800 units/mg; Worthington) and spleen phosphodiesterase (0.845 unit/mg; Worthington) according to the procedure of Josse *et al.* (15). The deoxyribonucleosides liberated from 3′-termini of DNA were separated from the 3′-deoxyribonucleotides by paper chromatography. A nuclease digest was concentrated to a small volume under an infrared lamp and spotted onto a sheet of Whatman No. 40 paper. In order to detect spots of deoxyribonucleosides under the ultraviolet lamp, 100 nmol each of F_3TdR and TdR was added as carriers. Ascending development (16) overnight using a solvent consisting of *n*-butanol:ethanol:water (50:15:35) provided spots of F_3TdR at R_f 0.82 and of TdR at R_f 0.62. The R_f values of all 3′-deoxyribonucleotides were under 0.40. The spots of nucleosides were cut out and placed in vials, and the radioactivity was measured by liquid scintillation counting. There was no formation of nucleosides from 3′-nucleotides.

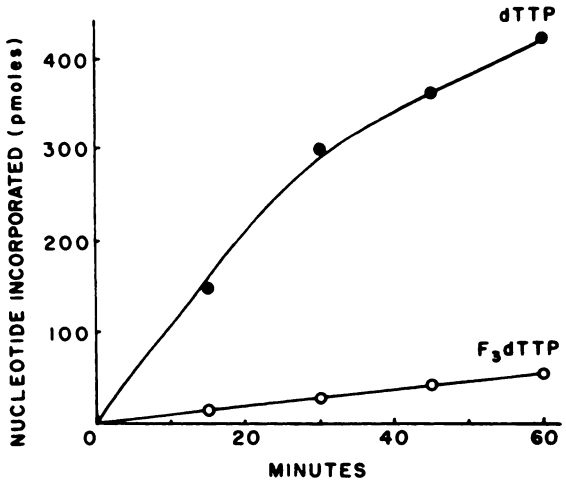

FIG. 1. *The incorporation of thymidine-5′-triphosphate and trifluorothymidine-5′-triphosphate into activated calf thymus DNA by HeLa cell cytoplasmic DNA polymerase α*

The reaction mixture (250 μl) contained 25 nmol each of doexyadenosine-5′-triphosphate, deoxyguanosine-5′-triophosphate, and deoxycytidine-5′-triphosphate, 50 μg of activated DNA, 250 nmol of 2-mercaptothanol, 10 μmol of Tris–HCl (pH 8.0), 2.5 units of DNA polymerase, and 12.5 nmol of [^{3}H]thymidine-5′-triphosphate (●) or 12.5 nmol of [^{3}H]trifluorothymidine-5′-triophosphate (○). The reaction mixture was incubated for the specified time at 37°C and assayed for the amount of radioactivity in the acid-insoluble fraction by the filter disk method as described previously (7).

The incorporation of [^{3}H]F_3dTTP and [^{3}H]dTTP into calf thymus DNA by the cytoplasmic DNA polymerase α is presented in Fig. 1. The incorporation of [^{3}H]F_3dTTP was linear with time up to 60 min, while the incorporation of [^{3}H]dTTP, the natural metabolite, was linear for about 30 min. A concentration of 12.5 nmol of either labeled nucleotide was added to the incubation mixture (250 μl). The maximum incorporation of [^{3}H]dTTP was approximately 500 pmol at 60 min, while the incorporation of [^{3}H]F_3dTTP into acid-insoluble form was less than 100 pmol.

When DNA is digested by spleen phosphodiesterase and micrococcal nuclease, the 3′-terminus is liberated as a nucleoside, since those nucleases hydrolyze the phosphodiester bonds of polynucleotide chains to form 3′-mononucleotides, except for the 3′-terminus which lacks a phosphate group at the 3′-position. Table 1 gives the results of the analyses. In Product A, containing 1180 pmol of [^{3}H]F_3dTTP, 7.95 pmol (0.67%) of [^{3}H]F_3TdR

TABLE 1

Analysis of 3′-termini of calf thymus DNA containing [^{3}H]F_3dTMP and [^{3}H]dTMP incorporated by HeLa cell cytoplasmic DNA polymerase

DNA preparation	[^{3}H]F_3dTTP (pmol)			[^{3}H]dTTP (pmol)		
	Amount added	Total incorporated	3′-Termini as nucleoside	Amount added	Total incorporated	3′-Termini as nucleoside
(A) [^{3}H]F_3dTTP	62,500	1,180	8.0 (0.67%)	—	—	—
(B) [^{3}H]F_3dTTP followed by [^{3}H]-dTTP	62,500	1,180	0.0 (0%)	6,250	1,890	7.5 (0.4%)
(C) [^{3}H]F_3dTTP concurrent with [^{3}H]dTTP	31,350	—	4.0	2,500	—	7.0

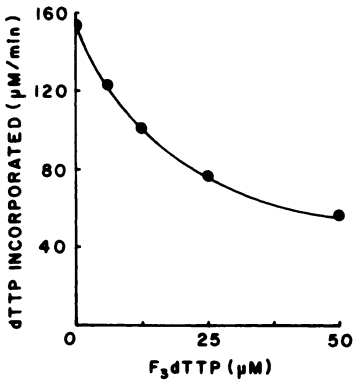

FIG. 2. *The effect of trifluorothymidine-5′-triophosphate on the incorporation of ^{3}H-thymidine-5′-triphosphate into calf thymus DNA by HeLa cytoplasmic DNA polymerase α*

The reaction mixture (125 μl) contained 6.25 nmol each of deoxyadenosine-5′-triphosphate, deoxyguanosine 5′-triphosphate, and 5′-deoxycytidine-5′-triphosphate, 2.5 nmol of [^{3}H]thymidine-5′-triphosphate, 25 μg of activated DNA, 1 μmol of $MgCl_2$, 125 μmol of 2-mercaptoethanol, 5 μmol of Tris–HCl buffer (pH 8.0), 2.3 units of enzyme, and the indicated concentration of trifluorothymidine-5′-triphosphate. The incorporation of [^{3}H]thymidine-5′-triphosphate was then determined as previously described (7).

was detected at the 3′-termini. Product B had 7.48 pmol (0.4%) of [^{3}H]TdR at its 3′-termini, but no 3′-terminus of [^{3}H]F_3TdR was detected. Since Product B was prepared by elongating the polynucleotide chain of Product A by incorporation of [^{3}H]dTTP and the other deoxyribonucleoside triphosphates, this observation demonstrates that the 3′-terminal F_3dTMP's, which originally existed in Product A, had disappeared. An additional 1890 pmol of [^{3}H]dTTP was incorporated after the prior incorporation of the 1180 pmol of [^{3}H]F_3dTTP.

By the enzymic digestion of Product C, 3.97 pmol of [^{3}H]F_3dTTP and 6.95 pmol of [^{3}H]dTTP were recovered as the 3′-termini. Although the maximal incorporation rate of dTTP was about 10 times higher than that of F_3dTTP (Fig. 1), in the incubation leading to Product C, the concentration of dTTP was reduced to a level at which only one-fifth of the maximal rate was obtained. Therefore, the amount of [^{3}H]dTTP recovered in the 3′-terminus was expected to be only twice that of [^{3}H]F_3dTTP, which is what we found (Table 1).

The results presented here clearly show that F_3dTTP is not a chain terminator when incorporated into DNA by DNA polymerase α, since less than 1% of the total amount of F_3dTTP was located at the 3′-termini. If F_3dTTP was a chain terminator, most, if not all, of the radioactivity would be located at the 3′-termini. Moreover, when [^{3}H]dTTP was used to elongate F_3dTMP-containing DNA (Product B), only [^{3}H]TdR was recovered at 3′-termini, yet the DNA obtained by simultaneous incorporation of [^{3}H]F_3dTTP and [^{3}H]dTTP contained both precursors at the 3′-termini (Product C). An important fact is that DNA containing F_3dTMP is an effective substrate for DNA polymerase α. Thus, the smaller sizes of cellular and viral DNAs obtained after exposure to F_3TdR (8, 9) are not caused by chain termination, and are the combined results of three factors: (i) F_3dTTP inhibits DNA polymerase both competitively with regard to dTTP and noncompetitively against the other nucleotides (7); (ii) F_3dTMP is a noncompetitive inhibitor of thymidylate synthetase (6); and (iii) the rate of incorporation of [^{3}H]dTTP into DNA by DNA polymerase as a function of the F_3dTTP concentration (Fig. 2) decreased from 153 to 58 μmol/min in the presence of 50 μM F_3dTTP. The reduction in dTTP incorporation may be responsible at least in part for the smaller values obtained for the sedimentation coefficients of F_3dTMP-containing DNA (8, 9).

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