IN VITRO SYNTHESIS OF ØX-174 SINGLE-STRANDED DNA

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SUMMARY: An in vitro single strand DNA synthesizing system has been developed from cells deficient in Kornberg DNA polymerase activity. When ØX-174 infected cells are gently lysed and sedimented through a sucrose gradient there is a peak of ^{3}H-TTP incorporating activity towards the upper region of the gradient that is not found in uninfected cells. The product of this activity is DNase sensitive and RNase resistant. DNA-DNA hybridization suggest that only ØX-174 single strand DNA is being synthesized.

INTRODUCTION: Three different modes of phage specific DNA synthesis have been identified in cells infected with the small, single strand DNA (ssDNA) bacteriophage ØX-174. The penetrating ssDNA first becomes double stranded (RF DNA) at specific sites on the membrane. This reaction presumably occurs via a host enzyme since protein synthesis is not necessary during its synthesis. At these sites more RF DNA molecules are synthesized (for a review see 1). Finally ssDNA is synthesized in the cytoplasm from a double stranded intermediate (2). Phage specific proteins appear to be involved in this reaction since amber mutants in 7 of the 9 cistrons identified by this laboratory synthesize either no ssDNA or smaller pieces of ssDNA when infecting a sus^{-} host (3,4).

The following research was initiated with the question--can the ssDNA synthesizing activity be isolated for in vitro studies on ssDNA replication? Though a previous in vitro ssDNA system has been reported (5), the recent discovery of a bacterial mutant, polA, greatly limited in Kornberg DNA polymerase activity (6), allows one to develop a system with a much lower background level of TTP incorporation by the supernatant fraction of artificially lysed cells.

The technique used for isolation was a modification of the procedure reported by Knippers and Strätling (7) when they examined DNA polymerase activity from membrane fractions sedimented through a sucrose gradient upon a

60% sucrose cushion. Attention in our work was concerned with the upper region of the sucrose gradient where the ssDNA synthesizing activity was found to be (2) As will be shown below, there is a clear peak of ^{3}H-TTP incorporating activity, and the TTP is incorporated only into ØX-174 DNA.

METHODS: A ØX-174 sensitive strain of the polA mutant, E. coli H560 (7) was provided by Dr. Douglas Smith. Cells were grown and infected in medium consisting of 10g Bacto tryptone and 5g of KCl per liter (made to 0.003 M $CaCl_2$) at 37°C. Exponentially growing cells were infected at a multiplicity of infection of 5 with an amber mutant in the lysis gene, N-11 (3), whose DNA synthesis is normal but which allows the infection process to be prolonged without lysis of the cells. Cells were sedimented 45 min after infection, washed with 20% sucrose in TEG [0.01 M tris-HCl, pH 7.3, 0.005 M ethylene glycol bis (B-aminoethyl-ether) N,N'-tetraacetic acid] and resuspended in TEG at 5 x 10^9 to 1.5 x 10^{10} cells/ml. Lysozyme was added to 50 μg/ml and the suspension was kept in an ice bath for 30 min. The suspension was then left at room temperature for 2 min before adding Brij 58 and $MgSO_4$ to 0.5% and 0.005 M, respectively. After 3-5 min, 1-ml of the suspension was layered over a SW41 gradient consisting of a 2-ml cushion of 60% sucrose under a 5-20% sucrose gradient in TKMM (0.01 M tris-HCl, pH 7.3, 0.1 M KCl, 0.005 M $MgSO_4$, and 0.0025 M β-mercaptoethanol). The gradient was spun at 40,000 rpm for 1 hr at 2°C. 0.35-ml fractions were collected from the bottom of the tube and 0.1-ml of the following reaction mixture was added to each fraction: in TKMM per 0.1-ml--5 nmoles of dATP, dGTP, dCTP, 50 nmoles of ATP and 1-μCi of ^{3}H-TTP (specific activity of 12-Ci/mM). The reaction was run at 35°C for 20 min. Samples were precipitated on Whatman GF/C filters with 6% CCl_3COOH and 1% $Na_4P_2O_7 \cdot 10H_2O$. DNA for hybridization was extracted with phenol and chloroform and ethanol precipitated. DNA-DNA hybridization followed the technique of Denhardt (8).

RESULTS: Cells were infected with ØX-174, gently lysed, and layered over a sucrose gradient. The incorporation of ^{3}H-TTP into the fractions of the sucrose gradient is shown in Figure 1A. Upon the 60% cushion at fractions 4-6

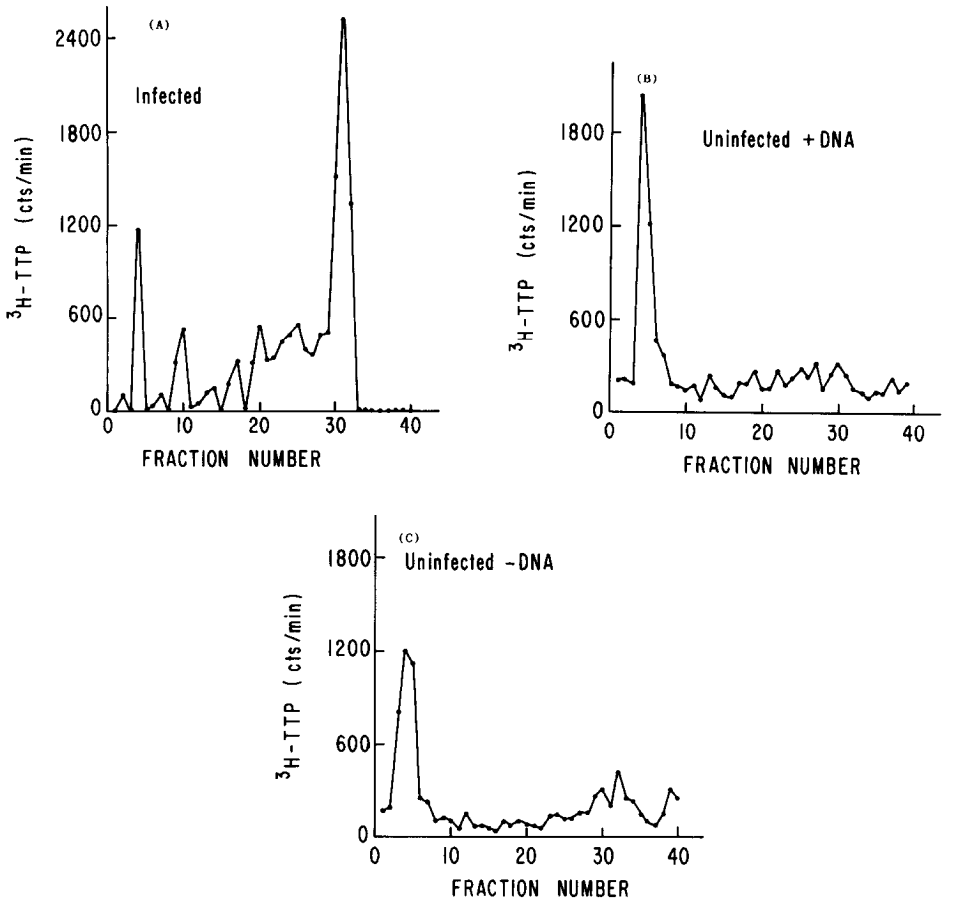

FIGURE 1: ^{3}H-TTP incorporation at 35°C for 20 min into sucrose gradient fractions of cells gently lysed and spun at 40,000 rpm for 1 hr at 2°C in an SW41 rotor. (A) infected cells; (B) uninfected cells with 5 μg calf thymus DNA added per reaction mixture; (C) uninfected cells without added DNA.

is the membrane activity reported by Knippers and Strätling (7). At fractions 30-32 there is a sharp peak of ^{3}H-TTP incorporation with which this paper is concerned. Phage particles normally sediment to fractions 14 and 15 in this gradient. The lesser peaks between the membrane fraction and fractions 30-32 (top activity) vary in position from gradient to gradient and have not been examined closely. ^{3}H-TTP incorporation into uninfected cells lysed and spun through the same type of gradient are shown in Figures 1B and 1C with and without calf thymus DNA added to their reaction mixtures, respectively. We have found no reproducible peak of ^{3}H-TTP incorporation at fractions 30-32 in uninfected cells.

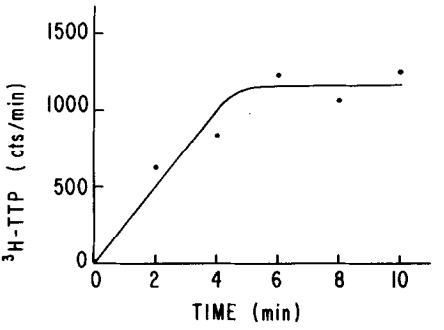

FIGURE 2: Five fractions about the top activity peak from infected cells were pooled and 0.5-ml of the reaction mixture was added. Incorporation of ^{3}H-TTP was measured at 35°C by pipetting out 0.3-ml aliquots every 2 min.

Kinetics of ^{3}H-TTP incorporation for the combined top activity fractions is shown in Figure 2. The incorporation appears to plateau between 4 and 6 min.

Preliminary evidence suggests that the absence or presence of ATP at the level given in the methods section has no effect upon the top activity though it normally has been added because of the reported positive effect it has upon the membrane bound DNA polymerase (7). The presence of 0.02 M EDTA in the reaction mixture reduces the incorporation to 2.4% suggesting that divalent cations are necessary. Table 1 shows that the product is DNase sensitive and RNase resistant.

To identify the type of DNA being synthesized, DNA-DNA hybridization was performed on the deproteinized product of the top activity fractions (Table 2).

TABLE 1: Sensitivity of the *in vitro* synthesized product to nucleases. Reactions were run with the top activity fractions as given in the methods section. DNase or RNase was added after 20 min and left for 1 hr at 35°C.

Complete system	100 %
30 μg/ml pancreatic DNase	10.1%
30 μg/ml pancreatic RNase	113.1%

Percent represents CCl_3COOH precipitable counts after nuclease digestion when compared to undigested ^{3}sample.

TABLE 2: DNA-DNA hybridization of the top activity product. Using the method of Denhardt (8) the *in vitro* synthesized product was hybridized with filters to which ssDNA, RF DNA, *E. coli* DNA and no DNA were affixed. Each horizontal row represents a separate experiment with cts/min bound to blank filters subtracted in each case (this background value was between 6-12 cts/min)

	Input cts/min	Cts/min bound to:		
		ssDNA	RF DNA	*E. coli* DNA
Expt. 1	630	3	226	21
Expt. 2	1320	2	540	16
Expt. 3	463	0	168	7

The lack of hybridization with ssDNA and *E. coli* DNA along with the high level of hybridization to RF DNA (approximately 35-40% of the input counts) suggest that only the positive strand (the strand found in the phage particle) is being synthesized.

DISCUSSION: The results have answered the question concerning the feasibility of isolating the ssDNA synthesizing activity. Figures 1A, 1B, and 1C show that the appearance of this activity requires ØX-174 infection. When infected cells are pulsed with ^{3}H-thymidine late in infection and the DNA deproteinized from the region of the top activity fractions, this DNA sediments slightly faster than RF DNA with a nick in it (RF II) and is presumably the ssDNA replicating intermediate reported by Knippers *et al.* (2). This suggests that the top activity represents a DNA-enzyme complex. That the DNA polymerase and template DNA may coincidentally sediment to the same region of the sucrose gradient in Figure 1A would be excluded by Figure 1B if one assumes the polymerase would work with calf thymus DNA.

If ssDNA is synthesized according to the rolling circle model of Gilbert and Dressler (9) then one would assume the ssDNA synthesizing complex to have a double stranded RF DNA form off which a ssDNA "tail" would come as ssDNA was being synthesized. ØX-174 structural proteins would be attached to this "tail" constraining the complex to form the ØX-174 particle during ssDNA synthesis. Presumably there would be a ssDNA polymerase of phage or host origin associated

with this complex functioning in concert with phage structural proteins. Our research is currently directed at investigating the components of our top activity fractions with the above considerations in mind.

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