JOURNAL OF VIROLOGY, Apr. 1975, p. 759-775

Vol. 15, No. 4
Printed in U.S.A.

In Vitro Polyoma DNA Synthesis: Inhibition by 1-β-D-Arabinofuranosyl CTP

TONY HUNTER AND BERTOLD FRANCKE*

The Armand Hammer Center for Cancer Biology, The Salk Institute, San Diego, California 92112

Received for publication 14 August 1974

The effects of 1-β-D-arabinofuranosyl CTP (ara-CTP) on DNA replication were studied in an in vitro system from polyoma-infected BALB/3T3 cells. Ara-CTP concentrations of ≥ 150 μM were found to block in vitro DNA synthesis completely, and concentrations of ≤ 0.3 μM had no inhibitory effect. Intermediate concentrations resulted in a concentration-dependent reduction of the in vitro synthesis rate. Long-term labeling with [α-^{32}P]ara-CTP demonstrated the incorporation of the analogue into cellular and viral DNA concomitantly with [^{3}H]TTP. In pulse-labeling experiments, at noninhibitory concentrations of the analogue, ara-CTP was incorporated into short DNA fragments and long growing strands to relatively the same extent as TTP. Partial venom phosphodiesterase digestion liberated the incorporated ara-CTP at essentially the same rate as incorporated TTP, excluding a predominantly terminal incorporation, and after total venom phosphodiesterase digestion >80% of the incorporated ara-CTP was recovered as 5'-ara-CMP. Analysis of the long-term in vitro viral DNA product made in the presence of partially inhibiting ara-CTP concentrations demonstrated that none of the steps leading to mature viral DNA were totally inhibited at the ara-CTP concentrations used. Pulse labeling of replicating viral DNA in the presence of ara-CTP revealed two consistent differences in the pattern found in control pulses: (i) predominant labeling of short chains (5*S*) with reduced amounts of radioactivity in the longer growing viral DNA strands ($\leq$16*S*), and (ii) a one-third to one-half reduction in size for short DNA chains labeled in the presence of ara-CTP. Release of the ara-CTP inhibition with excess dCTP resulted in covalent extension of these smaller short chains to approximately the size of regular short chains labeled in the absence of the inhibitor. Isolated short chains synthesized in the presence of ara-CTP exhibited a slightly lower degree of self-complementarity than regular short chains. The predominant labeling of short chains during pulses is, therefore, not a consequence of discontinuous growth on both sides of the replication fork. Similar results were obtained with ara-ATP and *N*-ethylmaleimide. The experiments indicate that ara-CTP acts primarily on DNA-polymerizing activities, affecting different DNA polymerases to varying degrees. The results are discussed in terms of the possible number and identity of polymerases involved in viral (and cellular) DNA replication.

An inhibitory effect of 1-β-D-arabinofuranosyl cytosine (ara-C) on the DNA synthesis and growth of *Escherichia coli* has been noted (22). In this organism, not all of the DNA-polymerizing processes are affected equally by ara-C. Repair-type synthesis is relatively resistant when compared to replicative synthesis (38). Inhibition studies on isolated enzymes have shown that *E. coli* DNA polymerase I is resistant to ara-CTP, whereas DNA polymerases II and III are sensitive to inhibition (38). For two of the known DNA polymerases in mammalian cells (39, 7, 35, 37), a similar distinction can be made on the basis of their sensitivity to ara-CTP (13; R. Fox and M. Goulian, personal communication). It is known that the synthesis of simian virus 40 and polyoma DNA is strongly inhibited by ara-C during viral infection in vivo (4). Bearing in mind the differential sensitivity of mammalian DNA polymerases to ara-CTP, we have used ara-CTP in an in vitro system derived from polyoma-infected mouse cells as a probe to dissect the DNA replication process into its components and to determine which DNA polymerases might be involved.

The in vitro system used for these studies has

been described (19). In vitro synthesis of viral DNA involves practically all of the replicative intermediates (RIs) present at the time of preparation of the lysate, and the majority of them are completed into mature viral DNA (form I). It has been shown (11) that synthesis in this system proceeds, at least in part, discontinuously, involving Okazaki-type DNA fragments (30) (5.3S) as precursors to long DNA chains, and that such fragments are initiated by an RNA primer (20). We have chosen this system for the present study because the pattern of discontinuous growth has been well established (11), such that any deviations from this pattern as a consequence of the added analogue should be readily recognizable. The results obtained under partially inhibitory concentrations of ara-CTP suggest that at least two distinguishable DNA-polymerizing activities are involved in DNA replication.

MATERIALS AND METHODS

Cells, virus, and culture conditions. BALB/3T3 cells were obtained from S. Aaronson. They were cultured in Dulbecco modified Eagle medium supplemented with 10% calf serum and infected with the polyoma ts1260 mutant as described (19).

In vitro system. Thirty-six hours after infection at 32 C, the cells were transformed to 39 C for 2 h, and a concentrated lysate was prepared as described previously (19). For in vitro DNA synthesis, the following additions were present (final concentrations in the reaction mixture): ATP, 1 mM; CTP, GTP, and UTP, 100 μM each; dATP, dCTP, dGTP, and TTP, 100 μM each (the unlabeled deoxynucleoside triphosphate corresponding to the labeled one was reduced to 20 μM for long-term incubations and left out completely for pulse-label experiments; dCTP was omitted in experiments with ara-CTP and the corresponding controls without ara-CTP); creatinine phosphate, 5 mM; creatine phosphokinase, 200 μg/ml; dithiothreitol, 1 mM; EGTA, 1 mM; *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid (pH 7.8), 40 mM; KCl, 100 mM; $MgCl_2$, 5 mM; $MnCl_2$, 1 mM; and $CaCl_2$, 0.5 mM. Variations on this incubation mixture will be mentioned for individual experiments. Incubation was at 32 C. Pulses were generally made 1 min after the start of the incubation period. The reaction was terminated by a modified Hirt extraction (17, 19). Determinations of incorporated radioactivity and the counting efficiency of ^3H and ^{32}P were as described previously (19).

Isolation of viral DNA and centrifugation techniques. Viral DNA was routinely purified by sedimentation through a neutral sucrose gradient from which the relevant fractions were pooled, concentrated by ethanol precipitation, and extracted with phenol (19). Purification of short (5*S*) DNA chains was as described by Francke and Hunter (11). Analyses of viral DNA by alkaline sucrose gradient sedimentation and equilibrium centrifugation in ethidium bromide containing CsCl, neutral, and alkaline Cs_2SO_4 gradients were described previously (19, 20).

Preparation of marker DNAs. ^3H-, ^{32}P- or ^{14}C-labeled viral marker DNAs (forms I and II) were prepared as described previously (19). The Hpa II fragments G and H were kindly provided by M. Vogt. They are the two smallest fragments of polyoma DNA produced by the restriction endonuclease II from *Haemophilus parainfluenzae* (a kind gift of J. Marrow, Stanford) and represent 5.2 and 1.8% of the total polyoma genome, respectively.

Gel electrophoresis. DNA samples were mixed with the markers and denatured by boiling for 2 min in a solution of 0.01 M Tris (pH 7.4) and 0.003 M EDTA (total volume 50 μl). After quick cooling in an ice bath, the sample was made 15% in glycerol and 0.01% in bromophenol blue. Electrophoresis was by the method of Edgell et al. (9) as modified by Huang et al. (18). Gels (12.5 cm, 7.5% polyacrylamide) were electrophoresed at 75 V for 6 h. An Autogeldivider (Savant) was used, and the fractions containing the crushed gel were counted in Triton-toluene (1:3) containing 3 g of 2, 5-diphenyloxazole per liter.

Venom phosphodiesterase digestion. DNA samples labeled with [^3H]TTP and [α-^{32}P]ara-CTP, denatured by boiling for 2 min in a solution of 0.01 M Tris (pH 7.4) and 0.001 M EDTA followed by quick cooling in an ice bath, were subjected to digestion by venom phosphodiesterase (100 μg/ml) in the presence of 100 μg of denatured calf thymus DNA per ml at 35 C under the conditions described previously (11). For partial digests, the reaction was terminated at the times desired by addition of 50 μg of yeast RNA and 5% trichloroacetic acid, followed by filtration onto Whatman GF/C glass-fiber filters. For complete digests, the reaction (1 h at 35 C) was continued for 4 h at 23 C and terminated by freezing (-20 C). Viral DNA was prepared for digestion as described above. Cellular DNA was prepared from Hirt pellets (see above) by a Marmur extraction (26) followed by ethanol precipitation. The precipitate was left to dissolve in a solution of 10 mM Tris-hydrochloride (pH 7.6) and 2.5 mM EDTA at 4 C for 2 days.

Chromatography. The complete venom phosphodiesterase digest was lyophilized and applied as a 1-cm streak to Whatman 3 MM paper, together with appropriate markers. Descending chromatography was performed in a solution of seven parts of 95% ethanol, three parts of 1 M ammonium acetate, and 1 mM EDTA for 24 h. The paper was dried and cut into 40 strips, which were then counted separately in a toluene-based scintillator.

Self-annealing. Purified 5*S* DNA was treated with 0.3 N KOH for 2 min at 100 C. Hybridization conditions and determination of amount of reannealing by resistance to the single-strand nuclease S1 (38) were as described (11). Results are expressed as a percentage of the total input counts of acid-precipitable material per minute after S1 digestion. Control samples, kept for the same length of time under hybridization conditions, and not treated with S1, indicated that no acid-precipitable radioactivity was lost during hybridization.

Chemicals. The sources of most chemicals were described earlier (19); ara-CTP and ara-ATP were from Terramarine Bioresearch; actinomycin D was from Calbiochem; venom phosphodiesterase (EC 3.1.4.1) was from Worthington (BAPF). S1 nuclease was a kind gift of M. Vogt. BrdUTP was synthesized by a modification of the Chamberlin-Berg method (6) or alternatively was purchased from Terramarine Bioresearch.

Radiochemicals. The source and specific activity of the ^{3}H-labeled deoxynucleoside triphosphates were as described previously (19). α-^{32}P-labeled deoxynucleoside triphosphates were purchased from New England Nuclear at about 100 Ci/mmol. [α-^{32}P]ara-CTP (200 Ci/mmol) was synthesized by the method of Biebricher (personal communication).

RESULTS

Effect of ara-CTP on the vitro synthesis kinetics of viral and cellular DNA. In vitro DNA synthesis in concentrated lysates of polyoma ts1260-infected 3T3 cells, as monitored by the incorporation of [^{3}H]TTP into acid-precipitable material, can be inhibited completely and instantaneously at any time during the reaction by the addition of 150 μM (or more) ara-CTP (19). This inhibition can be prevented by the simultaneous addition of a 10-fold excess of dCTP over ara-CTP. DNA, labeled before the ara-CTP block, was found to remain stable for at least 10 min in the presence of 200 μM ara-CTP. The addition of 0.3 μM ara-CTP (or less) had no measurable effect on [^{3}H]TTP incorporation. Since the pool of dCTP in the lysate is about 30 μM (19), it appears that a ratio of ara-CTP to dCTP of five is sufficient to inhibit DNA synthesis completely, whereas ratios of 1/100 or less are not inhibitory. At intermediate ara-CTP concentrations, the amount of inhibition is a function of the amount of ara-CTP added (Fig. 1). For both viral and cellular DNA, the initial rates during the first 10 min of linear incorporation are reduced, and for viral DNA the final plateau levels are also affected. The more pronounced leveling off of incorporation for viral DNA by comparison to cellular DNA is probably a consequence of the smaller size of the template and the inability of the lysate to initiate new rounds of replication (19).

Incorporation of labeled ara-CTP. One possible mechanism of inhibition by ara-CTP

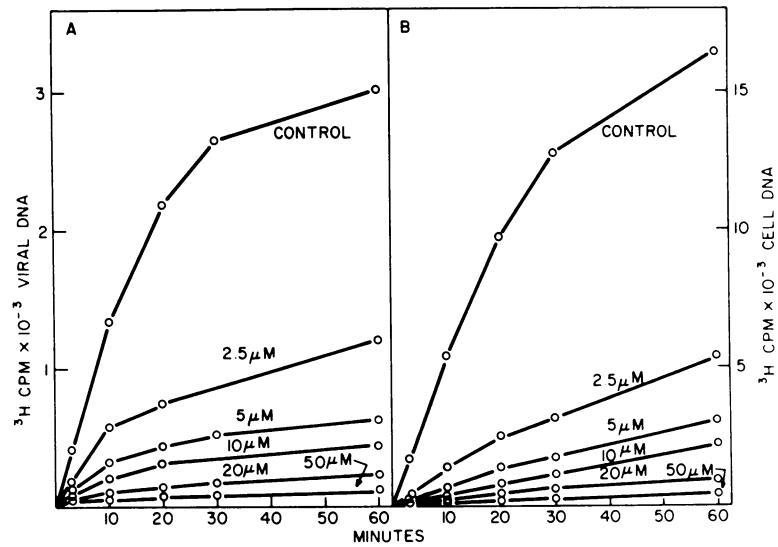

FIG. 1. *Time courses of in vitro synthesis of viral and cellular DNA in the presence of different concentrations of ara-CTP. Reaction mixtures (1.8 ml each) were prepared containing 0.9 volume of lysate from polyoma ts1260-infected BALB/3T3 cells and 0.1 volume of additions (TTP was reduced to 10 μM and dCTP was omitted); 20 μCi of [^{3}H]TTP (13.4 Ci/mmol) per ml was added. The mixture was divided in six parts (300 μl each), and ara-CTP was added at the concentrations indicated; 50-μl samples were incubated at 32 C for the times indicated, diluted with 1 ml of a solution containing 20 μM N-2-hydroxyethyl-piperazine-N'-2'-ethanesulfonic acid (pH 7.8), 3 mM EDTA, and 1 M NaCl, and brought to 1% SDS. After 10 min at room temperature, the samples were kept at 4 C overnight. After centrifugation for 30 min at 15,000 rpm and 4 C, 0.5 ml of the supernatant (viral DNA) was precipitated with 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate, and the acid-insoluble radioactivity was determined. The pellet (cellular DNA) was washed in 70% ethanol and solubilized in 1 N NaOH, and the acid-insoluble radioactivity in half the sample was determined. (A) Viral DNA; (B) cellular DNA.*

might be that it causes chain termination, either with or without terminal incorporation of the analogue, as suggested by Momparler (27). Graham and Whitmore (14), on the other hand, have provided evidence that, in vivo, base-labeled ara-C becomes incorporated into internucleotide linkages. Similarly, Brink and LePage (3) have reported incorporation of labeled ara-A. The following experiments were performed to examine the stability and incorporation of ara-CTP into DNA in the in vitro system.

Lysates were labeled simultaneously with [^{3}H]TTP and [α-^{32}P]ara-CTP either under noninhibiting conditions (0.3 μM ara-CTP) or with ara-CTP present at 10 μM. The time courses of incorporation of both labels into viral and cellular DNA are shown in Fig. 2. Concomitant incorporation of the two labels was observed over 60 min of incubation at both concentrations of ara-CTP. The α-^{32}P label from ara-CTP is incorporated into both viral and cellular DNA. Since the specific activity of the [α-^{32}P]ara-CTP was 30-fold lower at the higher ara-CTP concentration, it can be calculated that, relative to the [^{3}H]TTP incorporation, 20 times as much ara-CTP was incorporated into DNA at the higher ara-CTP concentration, even though there was an overall inhibition of DNA synthesis of 70%.

To test whether ara-CTP had been incorporated terminally or internally, DNA labeled in vitro with [^{3}H]TTP and [α-^{32}P]ara-CTP was subjected to digestion by venom phosphodiesterase. The time courses of digestion for both viral and cellular DNA, labeled in this manner under noninhibiting (0.3 μM) and inhibiting (10 μM) concentrations of ara-CTP, are presented in Table 1, which also contains a control time course for viral DNA labeled in the absence of ara-CTP. For the DNA synthesized under noninhibiting conditions, it is clear that both labels are liberated by the 3'-exonuclease at equal rates. The product synthesized in the presence of 10 μM ara-CTP also shows rather similar rates of release of the two labels, although there is a hint of an early release of ^{32}P. Thus, even under inhibiting conditions, the ara-CTP is not preferentially located at the 3' end of the product DNA. Hence, it appears that ara-C can substitute for dC, to a degree dependent on the relative concentrations of the precursors, at any location within the product strand.

To exclude the possibility that the α-^{32}P label of the ara-CTP was being reutilized after dephosphorylation, the nucleoside on the 5' side of the ^{32}P after incorporation of [α-^{32}P]ara-CTP was identified. A chromatographic analysis of a complete venom phosphodiesterase digest of the same cellular DNA as used for the partial

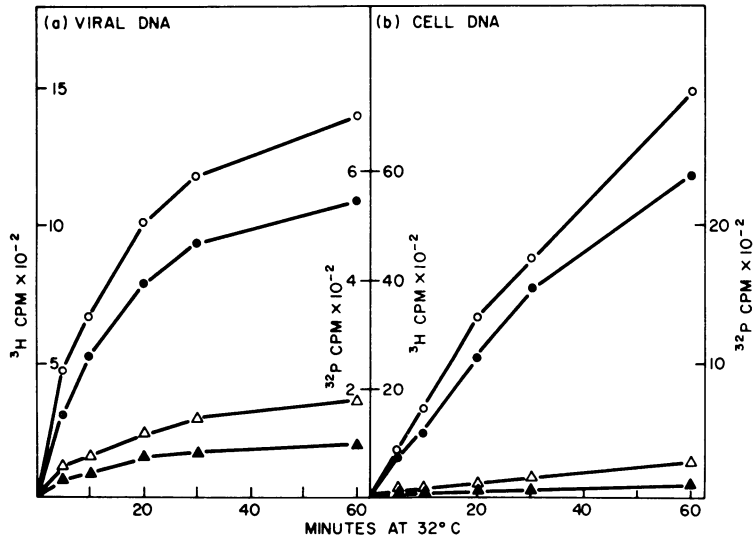

FIG. 2. *Time courses of [^{3}H]TTP and [α-^{32}P]ara-CTP incorporation into viral and cellular DNA at two different ara-CTP concentrations. Two 300-μl incubation mixtures were prepared (TTP was reduced to 10 μM and dCTP was omitted); 25 μCi of [^{3}H]TTP (13.4 Ci/mmol) and 50 μCi of [α-^{32}P]ara-CTP (200 Ci/mmol) per ml were added. The second incubation contained additional 10 μM unlabeled ara-CTP. Samples (50 μl) were incubated at 32 C. Experimental details were as described in legend to Fig. 1. (a) Viral DNA; (b) cellular DNA. Symbols: (○) [^{3}H]TTP and (●) [α-^{32}P]ara-CTP, 0.3 μM final ara-CTP concentration; (△) [^{3}H]TTP and (▲) [α-^{32}P]ara-CTP, 10 μM final ara-CTP concentration.*

digests (Table 1) was analyzed by chromatography. All of the ^{3}H radioactivity was found at the position of 5'-TMP, whereas 85% of the ^{32}P radioactivity comigrated with the 5'-ara-CMP marker. The remaining 15% corresponded to a breakdown product of ara-CMP that was also seen in the marker preparation. This probably arises through the spontaneous deamination of ara-CMP in solution, a process known to occur at least 40 times faster for ara-C than for dC (29). A less likely possibility is that it arises from the incorporation into DNA of ara-UTP that might be generated from ara-CTP in the in vitro incubation by pyrimidine deaminase (5). It is clear that the α-^{32}P label of the ara-CTP is not reutilized in the in vitro system and that ara-CTP can be incorporated as ara-CMP directly into DNA.

Effect of ara-CTP on the long-term in vitro viral DNA product. Since ara-CTP has a pronounced effect on the in vitro synthesis rate of viral DNA, it was of interest to determine whether any steps in maturation of viral RI to form I were blocked completely at partially inhibiting concentrations of the analogue. Viral DNA, labeled with [^{3}H]TTP in the absence or in the presence of 7.5 and 20 μM ara-CTP for 60 min at 32 C, was analyzed by alkaline sucrose gradient sedimentation to separate the rapidly sedimenting (53*S*) form I from the denaturable form II (labeled strand = 16*S*) and remaining RI (labeled strand $\leq$16*S*) (Table 2). Despite the high inhibition of total viral DNA synthesized at 20 μM ara-CTP, some form I was formed. It therefore appears that none of the processes leading to mature form I DNA is completely inhibited by 20 μM ara-CTP.

The properties of form I DNA labeled with [α-^{32}P]ara-CTP (0.3 μM) and [^{3}H]TTP were examined. After 60 min of incubation, the viral

TABLE 1. *Venom phosphodiesterase digestion of viral and cellular DNA labeled in vitro with [^{3}H]TTP and [$\alpha^{32}P$]ara-CTP*[a]

Ara-CTP (μM)	Digestion time (min)	% cpm[b] acid precipitable			
		Viral DNA		Cellular DNA	
		^{3}H	^{32}P	^{3}H	^{32}P
0	0	100 (= 3,279 cpm)			
	5	87			
	10	81			
	15	70			
	30	41			
	55	28			
0.3	0	100 (= 1,605 cpm)	100 (= 216 cpm)	100 (= 1,586 cpm)	100 (= 184 cpm)
	5	83	84	57	62
	10	74	76	27	28
	15	61	67	9	10
	30	32	38	4	[c]
	55	17	22	2	[c]
10	0	100 (= 600 cpm)	100 (= 133 cpm)	100 (= 1,295 cpm)	100 (= 359 cpm)
	5	79	65	85	71
	10	60	57	71	56
	15	51	45	62	52
	30	25	30	47	40
	55	17	20	24	25

[a] The DNA was labeled in 100-μl in vitro incubation mixtures (10 μM TTP, no additional dCTP) with 100 μCi of [^{3}H]TTP (17.4 Ci/mmol) per ml for 60 min at 32 C. One incubation mixture contained [$\alpha^{32}P$]ara-CTP (final concentration 0.3 μM, 200 Ci/mmol) and the other contained 1 μM [$\alpha^{32}P$]ara-CTP (brought to a final concentration of 10 μM with unlabeled ara-CTP). After extraction, the viral DNA (Hirt supernatant) and the cellular DNA (Hirt pellet) were treated separately with venom phosphodiesterase. Samples were removed from the digestion mixture at the times indicated, and the remaining acid-precipitable radioactivity was determined.

[b] cpm, Counts per minute.

[c] Too little counts per minute for a meaningful calculation of the percentage.

TABLE 2. *Analysis of viral DNA labeled with [^3H]TTP for 60 min in the presence of ara-CTP*[a]

Ara-CTP (μM)	Viral DNA synthesized in vitro		
	Total (% of control)	Form I (% of total)	Form II + RI (% of total)
0	100	55	45
7.5	16.7	20	80
20	7.7	16	84

[a] Viral DNA synthesized in 200-μl in vitro incubation mixtures, under the conditions described in legend to Fig. 1, was analyzed by preparative neutral sucrose gradient sedimentation. Total viral DNA was calculated as ^3H counts per minute sedimenting ≥16S to ≤30S (including form II, form I, and viral RI). The fractions corresponding to the total viral DNA were recovered and resedimented through alkaline sucrose gradients (5 to 20% sucrose in 0.25 N NaOH, total volume 3.5 ml) for 1.5 h at 55,000 rpm in a Beckman SW56 rotor at 5 C. Form I (53S) and denaturable DNA (form II and RI, ≤18S) are expressed as percentages of the total radioactivity present in each alkaline gradient.

DNA fraction (Fig. 3a) contained form I DNA as shown by its higher density in an ethidium bromide-cesium chloride equilibrium gradient (Fig. 3b). This form I sedimented rapidly in an alkaline sucrose gradient (Fig. 3c) and remained stable during treatment with 0.25 N alkali for 10 min at 60 C (Fig. 3d). Even though not all the form I molecules contain an ara-C residue under these labeling conditions, the $^3H/^{32}P$ ratio remained the same in all the fractionation procedures. We conclude that form I DNA containing ara-C residues has a supercoiled tertiary structure that denatures to a rapidly sedimenting form like unsubstituted form I viral DNA.

Effect of ara-CTP on in vitro pulse labeling of replicating viral DNA: (i) Distribution of label between short and long growing chains. If polyoma DNA is pulse labeled in vitro with [α-^{32}P]TTP for 1 min, beginning at 1 min after the start of incubation, most of the label is incorporated into viral RI (25S in neutral sucrose gradient sedimentation, Fig. 4a). The remainder of the radioactivity, which sediments

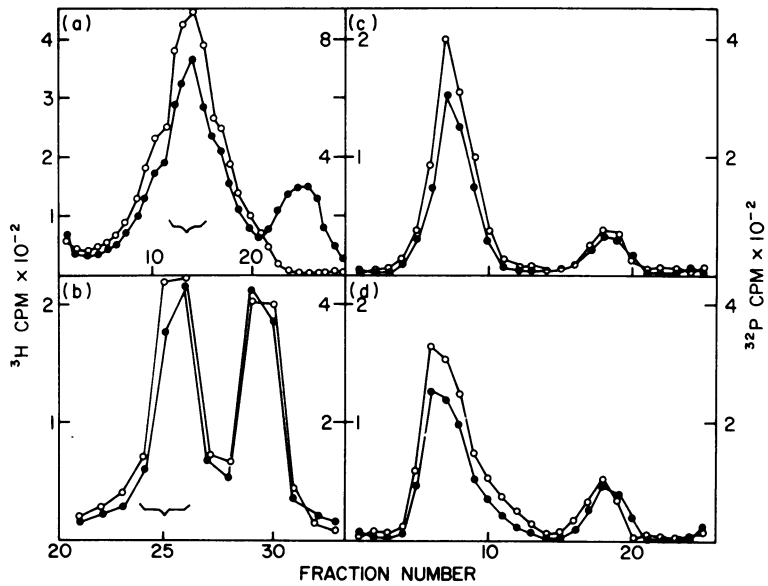

FIG. 3. *Analysis of viral DNA labeled for 60 min with [^3H]TTP and [α-^{32}P]ara-CTP. Reaction mixture (400 μl; containing 10 μM TTP and no dCTP) was incubated for 60 min at 32 C in the presence of 25 μCi of [^3H]TTP (13.4 Ci/mmol) and 50 μCi of [α-^{32}P]ara-CTP (200 Ci/mmol) per ml. The viral DNA fraction was sedimented through a neutral sucrose gradient, and 1/20 of each fraction was sampled for determination of radioactivity (a). The fractions corresponding to form I were recovered as indicated and centrifuged to equilibrium in an ethidium bromide-containing CsCl gradient, and 1/10 of each fraction was sampled (b). The fractions corresponding to supercoiled DNA were recovered as indicated and sedimented through alkaline sucrose gradients (Table 2, footnote a) without (c) and with (d) prior treatment with 0.25 N NaOH for 10 min at 60 C. From the alkaline gradients, total fractions were precipitated for determination of radioactivity. In these and all other gradient profiles shown, the top of the centrifuged tube corresponds to the right and the bottom corresponds to the left of the diagrams. (a) Neutral sucrose gradient; (b) ethidium bromide-CsCl gradient; alkaline sucrose gradient before (c) and after (d) alkali treatment. Symbols: ○, ^3H counts per minute; ●, ^{32}P counts per minute.*

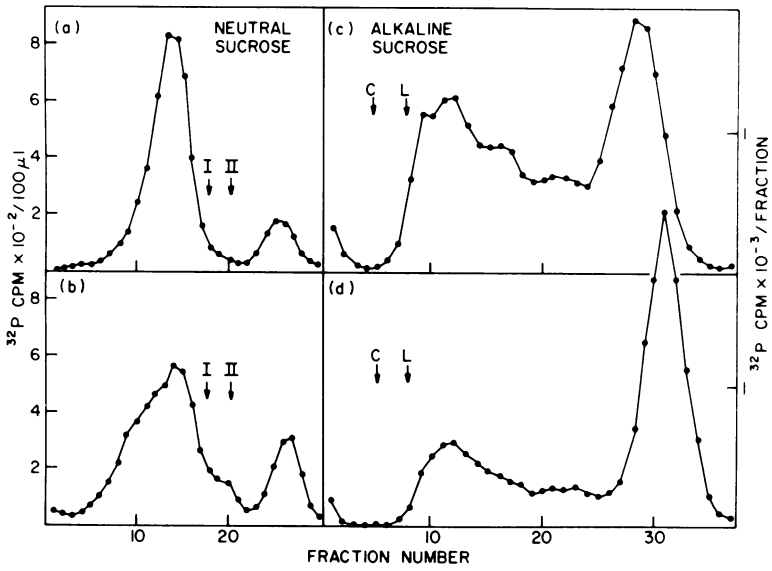

FIG. 4. *Analysis of viral DNA pulse labeled with [α-^{32}P]TTP in the absence and presence of 7.5 µM ara-CTP. Two reaction mixtures of 300 µl each were prepared (TTP and dCTP were omitted), one of them containing 7.5 µM ara-CTP. They were incubated for 1 min at 32 C and then pulse labeled with 100 µCi of [α-^{32}P]TTP (102.4 Ci/mmol) per ml. The pulse was terminated after 1 min in the absence and after 5 min in the presence of ara-CTP. The viral DNA fractions were mixed with ^{3}H-labeled polyoma DNA (forms I and II as markers) and sedimented through neutral sucrose gradients, and 1/20 of each fraction was sampled for determination of radioactivity (a,b). Fractions 6 through 20 (viral RI) were recovered, concentrated, and sedimented through alkaline sucrose gradients for 5.5 h at 55,000 rpm (c,d). The marker DNA peaks I and II and the circular (C) and linear (L) strands of form II in alkali are indicated by arrows. (a) 1-Min control pulse and (b) 5-min pulse at 7.5 µM ara-CTP: neutral sucrose gradients of the Hirt supernatant fraction. (c) 1-Min control pulse and (d) 5-min pulse at 7.5 µM ara-CTP: alkaline sucrose gradient of viral RI.*

near the top of the gradient, represents single-stranded DNA fragments, of mostly cellular origin, thought to detach from growing forks during extraction (11). If the fractions corresponding to viral RI are recovered from the gradient and subjected to a sedimentation analysis under denaturing conditions (alkaline sucrose gradient), the profile shown in Fig. 4c is obtained. The two size classes of DNA chains apparent in this profile represent the long growing chains (heterogeneous up to 16S, the size of unit length linear viral DNA) and the short DNA chains (5.3S) involved in discontinuous growth. The results of a similar analysis of viral DNA, pulse labeled in the presence of 7.5 µM ara-CTP, are presented in Fig. 4b (neutral gradient) and Fig. 4d (alkaline gradient). Because of the reduced rate of DNA synthesis in the presence of ara-CTP, the pulse length was extended to 5 min to obtain comparable amounts of DNA synthesized during the labeling period. The neutral gradients consistently showed an increase of labeling of the free single-stranded DNA fragments if the incubation was made in the presence of ara-CTP (cf. Fig. 4a and b). This is most likely because the smaller size of short DNA chains (see below) facilitates their detachment during extraction; however, detachment during the incubation, due to a slower rate of synthesis, might also contribute to this increase. A striking difference in the distribution of label between short and long growing strands is apparent in a comparison of the two alkaline gradients (Fig. 4c and d). The amount of radioactivity in long chains is decreased from 57 to 38% and the amount in short chains is increased from 43 to 62% of the total pulse label with 7.5 µM ara-CTP present. In addition, the position of the peak of short chains indicates that they are shorter in the inhibited case. The distribution of label in Fig. 4c suggests that ara-CTP inhibits the labeling of long chains more strongly than that of short chains.

To test whether this differential effect of ara-CTP might also be reflected by the incorporation of the analogue, the experiment illustrated in Fig. 5 was performed. Viral DNA was labeled with [^{3}H]TTP and [α-^{32}P]ara-CTP for 1 min at 0.5 µM ara-CTP (a, d) and 5 µM ara-CTP

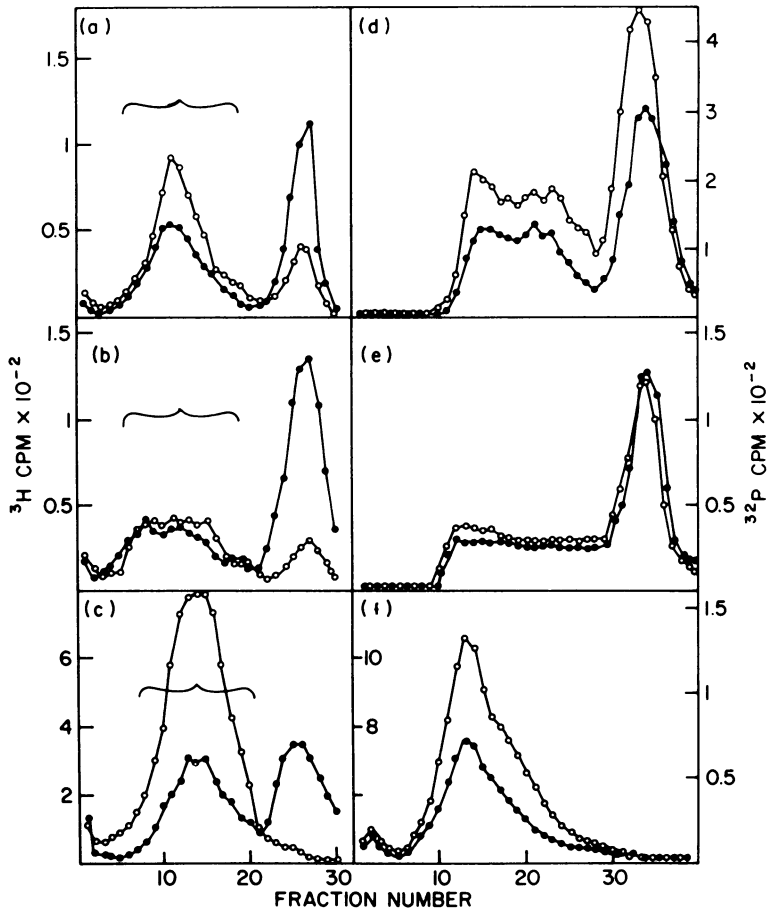

FIG. 5. *Analysis of viral DNA pulse labeled with [^{3}H]TTP and [α-^{32}P]ara-CTP and chased with excess dCTP. Three 400-μl reaction mixtures (no added dCTP or TTP) were pulse labeled with 50 μCi of [^{3}H]TTP (13.4 Ci/mmol) and 100 μCi of [α-^{32}P]ara-CTP (200 Ci/mmol) per ml starting at 1 min after the beginning of the incubation at 32 C. One pulse, containing no additional ara-CTP, was terminated after 1 min; the second, containing 5 μM ara-CTP, was terminated after 5 min; and the third, pulsed for 5 min in the presence of 5 μM ara-CTP, was further incubated for 10 min in the presence of 500 μM dCTP. Unlabeled TTP (10 μM) was also present during the chase, resulting in continued [^{3}H]TTP labeling during the chase at a lower specific activity than during the pulse. Further experimental details were as described in legend to Fig. 4. (a) 1-Min pulse (ca. 0.5 μM ara-CTP), (b) 5-min pulse (5 μM ara-CTP), and (c) same as (b) followed by 10-min chase with 500 μM dCTP: preparative neutral sucrose gradients. (d) 1-Min pulse (ca. 0.5 μM ara-CTP), (e) 5-min pulse (5 μM ara-CTP), and (f) same as (e) followed by 10-min chase with 500 μM dCTP: alkaline sucrose gradients of viral RI. Symbols: (○) ^{3}H counts per minute; (●) ^{32}P counts per minute.*

(b, e) (a and b represent the neutral gradients; d and e represent the alkaline gradients). The ratios of the two isotopes in long and short chains is the same (for both concentrations of the analogue), indicating that ara-C substitution occurs to the same degree for both size classes of DNA chains, although the distribution of label between the long and short chains is affected to a similar degree at the higher concentration of ara-CTP (Fig. 4).

(ii) Chase of pulse-labeled short chains in the presence of excess dCTP. Analysis under denaturing conditions of the long-term in vitro viral DNA product synthesized in the presence of 7.5 μM ara-CTP failed to demonstrate the presence of short DNA chains. Thus, the preferential labeling of short chains during pulses does not result in accumulation of such chains in the final product. To follow the joining of short chains under normal synthesis conditions, RI pulse labeled with [α-^{32}P]dCTP for 1 min in the absence and for 5 min in the presence of 7.5 μM ara-CTP was chased with 200 μM dCTP for different lengths of time. DNA synthesis during

the chase was monitored by the incorporation of [^{3}H]TTP. The alkaline sucrose sedimentation profiles of the purified RIs are presented in Fig. 6. In both cases, the ^{32}P pulse label present as short chains chases quantitatively into the larger size class. (The reduced rate of [^{3}H]TTP incorporation during the chase after a pulse in the presence of ara-CTP is probably due to a decrease in the efficiency of DNA synthesis after prolonged exposure to ara-CTP.) Fig. 6f shows an alkaline sucrose gradient profile of a similar chase of RI that had been pulse labeled with [^{3}H]TTP and [α-^{32}P]ara-CTP (5 μM), documenting a quantitative chase of ^{32}P radio-

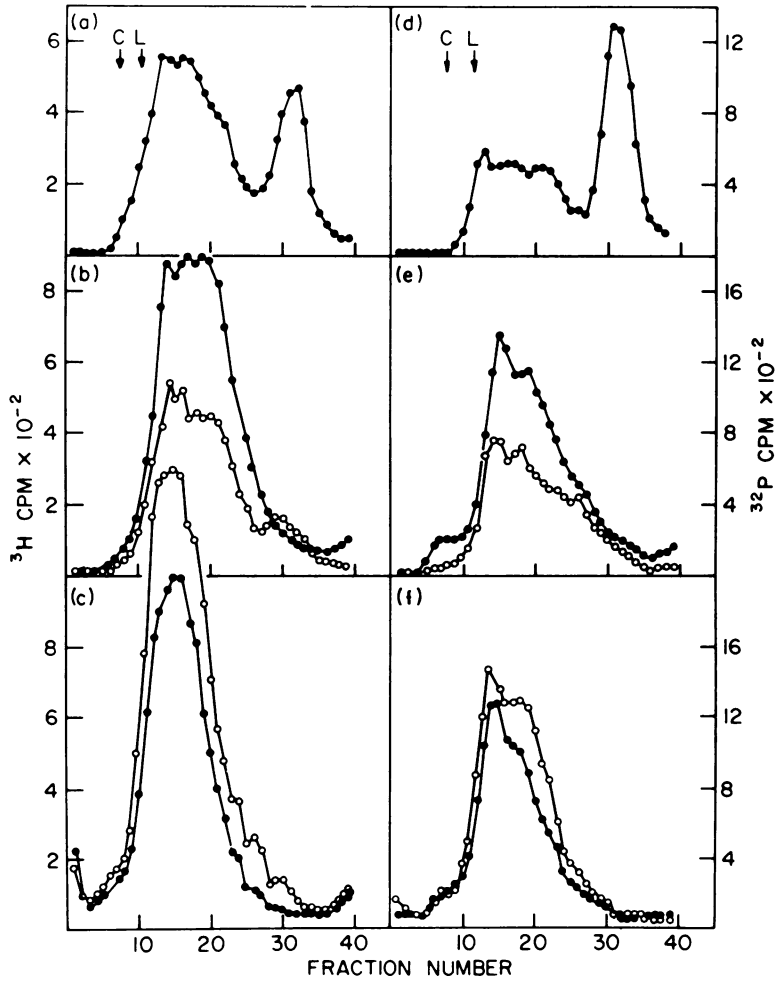

FIG. 6. *Alkaline sucrose gradient profiles of viral RI pulse labeled in the absence or presence of 7.5 μM ara-CTP and chased in the presence of [^{3}H]TTP and excess dCTP. Two 900-μl reaction mixtures (10 μM TTP, no additional dCTP) were pulse labeled with 70 μCi of [α-^{32}P]dCTP (100 Ci/mmol) per ml starting 1 min after the beginning of the incubation at 32 C. From the first sample (control), 300 μl was removed and extracted after the 1-min pulse (a); to the remainder, 500 μM dCTP and 60 μCi of [^{3}H]TTP (13.4 Ci/mmol) per ml were added, and two more 300-μl samples were taken at 3 min (b) and 10 min (c) after the beginning of the chase. The second sample (containing 7.5 μM ara-CTP from the beginning of the reaction) was pulse labeled for 5 min (d) and chased under the same conditions as the control for 5 min (e) and 15 min (f). Viral RI, purified by neutral sucrose gradient sedimentation (compare legend to Fig. 4), was analyzed by alkaline sucrose gradient sedimentation for 5.5 h at 55,000 rpm. The two samples taken directly after the pulses contained ^{3}H-labeled marker DNA, as described in legend to Fig. 4. (a) 1-Min pulse (control); (b) same as (a), chased for 3 min; (c) same as (a), chased for 10 min; (d) 5-min pulse (in the presence of 7.5 μM ara-CTP); (e) same as (d), chased for 5 min; (f) same as (d), chased for 15 min. Symbols: (O) ^{3}H counts per minute (label during chase); (●) ^{32}P counts per minute (pulse label).*

activity from the 5S region. Thus, short DNA fragments synthesized in the presence of ara-CTP and containing the analogue can serve as normal precursors to long viral DNA strands.

(iii) Self-annealing of short DNA chains. As reported previously (11), even during very short pulses, the in vitro system incorporates at least 50% of the label into long strands ($\leq$16S). The interpretation of this finding in terms of a semi-discontinuous mode of synthesis in this system was supported by low self-annealing levels of isolated short chains. A possible reason for the altered labeling pattern during pulses in the presence of ara-CTP might therefore be that the discontinuous mode of chain growth, which in the absence of the analogue is restricted to one side of the growing fork, operates on both sides. If this were the case, isolated short DNA chains synthesized in the presence of the inhibitor should exhibit a higher degree of self-complementarity than under non-inhibited conditions.

Table 3 shows the time courses of self-annealing of short DNA chains from viral RI, labeled in the absence or presence of 7.5 μM ara-CTP. To avoid the contamination with randomly broken preexisting viral DNA, the in vitro reactions were carried out in the presence of BrdUTP. The short chains labeled with [α-^{32}P]dGTP were isolated after denaturation of the RI by neutral sucrose gradient sedimentation and purified by equilibrium centrifugation in alkaline Cs_2SO_4. Greater than 80% of the ^{32}P label was found to band at the position of dense DNA. The density distribution was the same whether ara-CTP was present or not, indicating that the analogue does not interfere with the initiation of short chains.

The self-annealing of such fragments at plateau levels is slightly lower if 7.5 μM ara-CTP was present during their synthesis. This difference is probably not significant. The result does not support a switch to discontinuous chain growth on both sides of the growing fork in the presence of the analogue, but rather suggests a preferential inhibition of the continuously growing strand as an explanation for the altered pulse-labeling pattern.

(iv) Size of short DNA chains. As suggested by the alkaline gradients shown in Fig. 5b and d, the short chains made in the presence of ara-CTP appear to be shorter than in the control. To confirm this observation and to obtain quantitative measurements of the difference, short chains were analyzed by polyacrylamide gel electrophoresis. The markers used for the gels shown in Fig. 7 were the fragments G and H, produced by the restriction endonuclease HpaII from polyoma DNA (^{3}H labeled). They constitute the two smallest fragments of the eight unique fragments generated by this enzyme (16). The molecular weight of fragment G (5.2% of the genome) is 8.85×10^4 and that of fragment H (1.8% of the genome) is 3.06×10^4 after denaturation into single strands (full-length polyoma single strand mol wt = 1.7×10^6). Both fragments migrate as narrow bands (Fig. 7), indicating their homogeneity, whereas the profiles for short chains from viral RI show a wider spread. Nevertheless, the positions of the peaks are clearly different, demonstrating the smaller size of short chains labeled in the presence of 7.5 μM ara-CTP (Fig. 7b). For the electrophoresis conditions used (18), the linear relationship between the log of molecular weight and distance migrated for single-stranded DNA in this size range has not yet been established. Assuming such a relationship and using the fragments G and H and bromo-

TABLE 3. *Self-annealing of short DNA chains labeled in the absence or presence of ara-CTP*[a]

Labeling conditions	Time of hybridization (h)	Resistance to SI[b] (%)
1. [α-^{32}P]dGTP for 2 min at 32 C	0	2.3
	3	7.8
	24	25.2
	48	29.6
2. [α-^{32}P]dGTP for 10 min at 32 C in the presence of 7.5 μM ara-CTP	0	1.3
	3	7.3
	24	20.8
	48	23.3

[a] 1.0 ml of incubation mixture (150 μM BrdUTP, no added dCTP, dGTP or TTP) was pulse labeled for 2 min with 85 μCi of [α-^{32}P]dGTP (control) or for 10 min in the presence of 7.5 μM ara-CTP. Short chains were purified from viral RI after heat denaturation by sedimentation through neutral sucrose gradients. The fractions corresponding to short chains were concentrated and centrifuged to equilibrium in alkaline Cs_2SO_4 gradients along with ^{3}H-labeled viral form II DNA as a marker for "light" density. The fractions corresponding to "heavy" density were dialyzed against a solution of 0.01 M Tris (pH 8.0) and 0.003 M EDTA, concentrated to 200 μl by precipitation with ethanol, and hybridized. The amount of double-stranded DNA formed is expressed as percent resistance to the single-strand-specific nuclease SI. Control hybridization to polyoma DNA gave >95% resistance to SI for both types of short chains. No acid-precipitable counts per minute were lost during 60 h at 68 C, as indicated by control samples not treated with SI.

[b] For conditions 1 and 2, 100% resistance equals 218 and 573 counts/min, respectively.

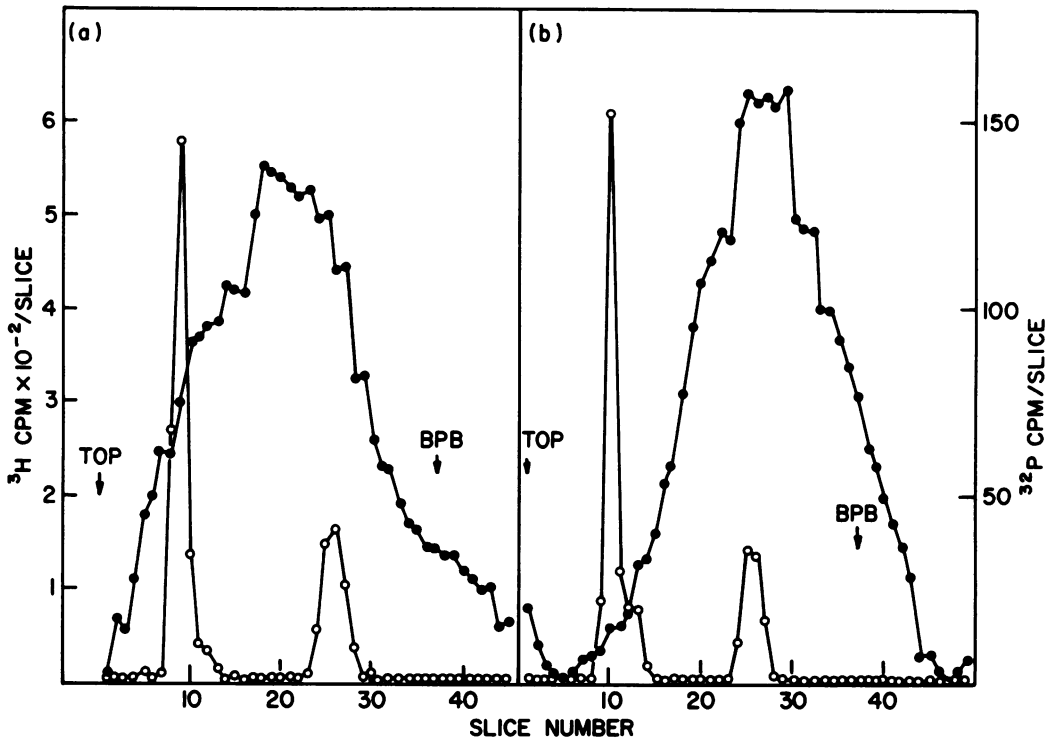

FIG. 7. *Co-electrophoresis on polyacrylamide gels of short DNA chains pulse labeled with [α-^{32}P]TTP in the absence and presence of 7.5 μM ara-CTP, with denatured Hpa II fragments G and H. In vitro pulse labeling was as described in legend for Fig. 4. Purified viral RI was denatured by boiling for 2 min followed by quick cooling in an ice bath. After sedimentation through a preparative neutral sucrose gradient, the fractions corresponding to short chains were recovered and concentrated. After treatment with 0.25 M NaOH for 2 min at 60 C, followed by neutralization with HCl, the samples were concentrated again and mixed with ^{3}H-labeled Hpa II fragments G and H in 50 μl of 1:100 SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate). After heat denaturation (100 C, 2 min), the samples were chilled and electrophoresed. Fractions corresponding to 2.5 mm of gel were counted for radioactivity. Electrophoresis was from left to right. (a) 1-Min pulse (control), (b) 5-min pulse (in the presence of 7.5 μM ara-CTP). Symbols: O, ^{3}H counts per minute (Hpa II fragments G and H); ●, ^{32}P counts per minute (pulse-labeled short chains from viral RI).*

phenol blue as standards, the plot presented in Fig. 8 was constructed. The sizes for short DNA chains obtained from this plot are listed in Table 4. Apart from the average size obtained from the peak maximum, Table 4 also includes the band width for half-peak height as a rough estimate of the heterogeneity. These figures are not the statistical mean deviation since the profiles do not show a Gaussian distribution.

When short fragments were analyzed after a 1-min pulse had been followed by a 30-s chase, their size had slightly increased and they formed a narrower band in the gel. This value would correspond to the maximum length to which short chains can grow shortly before their joining to long strands. Short fragments, found in the free single-stranded DNA fraction near the top of neutral sucrose gradients (compare Fig. 4a), are considerably smaller, which might be one of the reasons for their preferential loss from the growing fork. The size of short DNA chains contained in viral RI labeled in the presence of ara-CTP corresponds to about two-thirds that of regular pulse-labeled short chains or to about one-half if compared to "full size" short chains after a 30-s chase.

Since all short chains present in the RI after pulse-labeling with ara-CTP can be eventually chased into longer chains with excess dCTP (compare Fig. 5e), it was of interest to determine whether they required elongation to regular full size before joining. This was tested in two ways. Figure 9a shows a gel electrophoresis profile of isolated short chains from viral RIs after pulse labeling for 1 min with [^{3}H]TTP in the absence of 7.5 μM ara-CTP and for 5 min with [α-^{32}P]dCTP in the presence of 7.5 μM ara-CTP. The difference in the size distribution

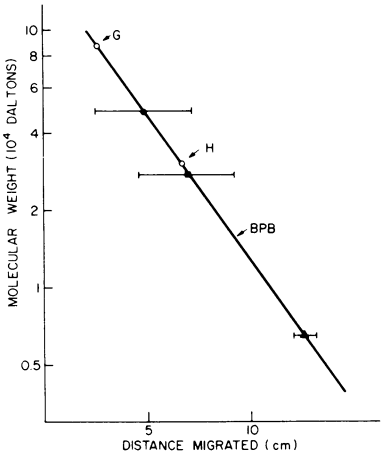

FIG. 8. *Estimation of the molecular weight of short DNA chains. The plot was constructed by assuming a linear relationship between molecular weight (plotted on a logarithmic scale) and the distance migrated. ^{32}P-labeled short chains were electrophoresed with denatured Hpa II fragments G and H and bromophenol blue as markers, as described in legend for Fig. 7. Peak maxima: (●) short chains from viral RI (pulse-labeled for 1 min), (■) short chains from viral RI (pulse-labeled for 5 min in the presence of 7.5 μM ara-CTP), and (▲) free single-stranded DNA fragments after a 1-min pulse (fractions 22 through 28 from Fig. 4a). (——) half-peak height.*

TABLE 4. *Sizes of various short DNA chains*[a]

Source of DNA	Size (number of bases)	
	Avg (peak maximum)	Mean variation (half-peak width)
Markers:		
Hpa II fragment G, denatured	295	
Hpa II fragment H, denatured	102	
Short chains from polyoma RI:		
Pulse labeled in vitro for 1 min	162	88–295
Same, chased for 30 s	221	140–333
Labeled in vitro for 5 min in the presence of 7.5 μM ara-CTP	92	52–175
Free single-stranded fragments, labeled in vitro for 1 min	22	18–25

[a] Gel electrophoresis was as described in the legend for Fig. 7, and the size of the DNA was determined by the method described in the legend for Fig. 8.

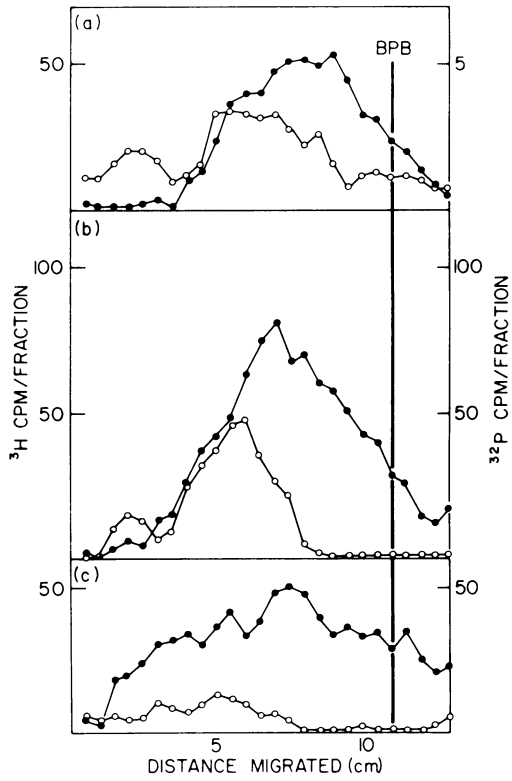

FIG. 9. *Gel electrophoresis of short DNA chains from viral RI labeled in the absence or presence of 7.5 μM ara-CTP and chased for different times in the presence of excess dCTP. Two 600-μl reaction mixtures were prepared. One was pulse labeled for 1 min with 62.5 μCi of [^3H]TTP (13.4 Ci/mmol) per ml (no additional TTP in the reaction), and 200 μl was removed and extracted (a). The remainder was chased with 500 μM TTP and 500 μM dCTP for 30 s (b) and 60 s (c). The other incubation (containing 7.5 μM ara-CTP, no additional dCTP) was pulse labeled for 5 min with 75 μCi of [α-^{32}P]dCTP (100 Ci/mmol) per ml (a) and chased for 30 s (b) and 60 s (c) in the presence of 500 μM TTP and 500 μM dCTP. The viral DNA fractions (Hirt supernatants) were pooled from the two pulsed samples, the two 30-s chase times, and the two 60-s chase times, respectively. Short chains were prepared from purified viral RI as outlined in legend to Fig. 7. Electrophoresis was as described in legend to Fig. 7, except that no markers were added and fractions corresponding to 5-mm gel length were counted for radioactivity. (a) Short chains immediately after the pulse labeling, (b) after 30 s, and (c) after a 60-s chase with excess dCTP. Symbols: O, ^3H counts per minute (TTP pulse in the absence of ara-CTP); ●, ^{32}P counts per minute (dCTP pulse in the presence of 7.5 μM ara-CTP).*

resembles that in Fig. 7. If the chains were chased with excess dCTP and TTP for 30 s and 1 min, the profiles shown in Fig. 9b and c were obtained. For the ^3H-labeled chains, an initial sharpening of the band with a shift to longer sizes can be observed, followed by their disappearance. The latter is due to their joining to long chains, removing them from the 5S region

of the sucrose gradient employed for their purification. The ^{32}P-labeled chains also gain in size (Fig. 10b). The distribution resembles that of the ^{3}H profile in Fig. 9a. After 1 min (Fig. 9c), a portion of them is removed from the 5S DNA fraction, presumably due to joining, while the remainder is spread over a wide range of sizes, indicating that the completion of short chains after release of ara-CTP inhibition by dCTP is not a synchronous event. In the second experiment, short chains labeled for 1 min in the absence and for 5 min in the presence of 7.5 μM ara-CTP with [^{3}H]dATP were chased for 30 s and for 1 min with excess dCTP in the presence of BrdUTP. The density profiles in alkaline Cs_2SO_4 equilibrium gradients are shown in Fig. 10. For the chains labeled without ara-CTP present (Figs. 10a and b), some trailing toward higher densities can be observed after the 30-s chase, whereas after the 60-s chase they started disappearing from the 5S DNA fraction. For the chains that were labeled with the analogue present 30 s after the chase, a shoulder is observed at a density corresponding to about one-third of the density difference between fully light and fully BrdU-substituted DNA. This amount of substitution is in agreement with the amount of elongation that would be required to complete chains of the ara-CTP-inhibited size for the full size of regular short chains. At 60 s after the chase, the greater density shift is seen, suggesting that chains which were even shorter are being elongated as well, while a considerable amount still remains unelongated at fully light density. The experiments illustrated in Fig. 9 and 10 suggest that the smaller class of short chains made in the presence of ara-CTP is elongated by roughly the size difference between them and "regular" short chains when the inhibition is reversed with dCTP, implying that their shorter size is not a consequence of a closer spacing on the template strand. It is more likely that their spacing is the same as for regular short chains, but that they are separated by gaps that are filled in by elongation of the shorter chains after dCTP reversal. This gap-filling process does not appear to involve all chains synchronously at the time of reversal.

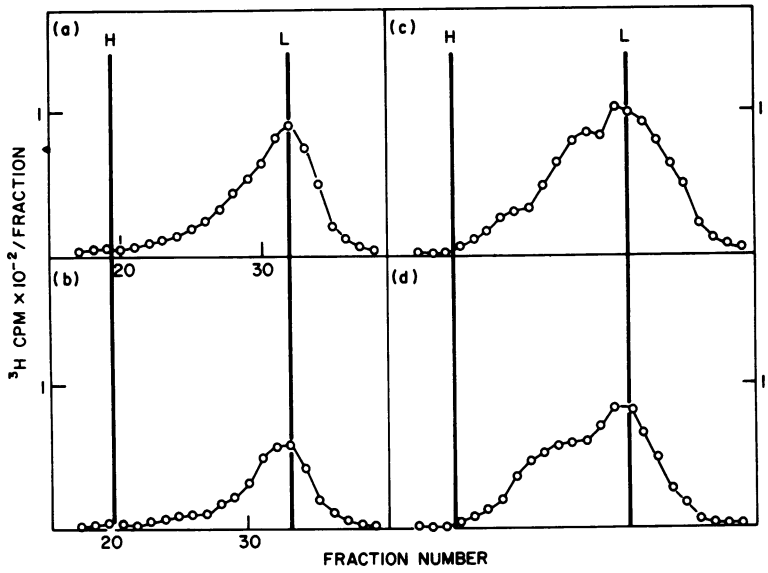

FIG. 10. *Alkaline Cs_2SO_4 gradient profiles of short chains from pulse-labeled viral RI chased with excess dCTP in the presence of BrdUTP. Two 600-μl incubation mixtures (no additional TTP, dATP, or dCTP) were pulse labeled with 60 μCi of [^{3}H]dATP (16.8 Ci/mmol) per ml, the control for 1 min and the other for 5 min, in the presence of 7.5 μM ara-CTP at 32 C. Both pulses were chased in the presence of 500 μM dATP, 500 μM dCTP, and 150 μM BrdUTP. After addition of the chase, the reactions were divided into two, and incubations were continued for 30 and 60 s, respectively. Short chains were purified as described in the legend to Fig. 7, mixed with ^{32}P-labeled viral form II DNA (light) and an equal amount of ^{32}P-labeled fully BrdU-substituted viral form II DNA (heavy) as markers, and centrifuged to equilibrium in alkaline Cs_2SO_4 gradients. Of the 60 fractions collected, only those containing the labeled DNA are shown. The positions of the ^{32}P marker DNAs are indicated by vertical lines (L, light; H, heavy). (a) 1-Min pulse (control) and 30-s chase, (b) 1-min pulse (control) and 60-s chase, (c) 5-min pulse (7.5 μM ara-CTP) and 30-s chase, (d) 5-min pulse (7.5 μM ara-CTP) and 60-s chase.*

Effect of other inhibitors of DNA replication on in vitro pulse labeling of viral DNA. The rather specific changes in the pulse-labeling pattern of viral RI due to the presence of ara-CTP in the reaction mixture suggests that a specific polymerase(s) might be affected by the analogue. To compare this pattern with that obtained for pulse labels in the presence of other inhibitors of DNA synthesis, the compounds ara-ATP (10 μM) and *N*-ethylmaleimide (NEM) (4 mM) were tested. Although normally regarded as an inhibitor of RNA synthesis, actinomycin D at high concentrations (10 μg/ml) also inhibits polyoma DNA synthesis in vitro (T. Hunter and B. Francke, unpublished data) and was tested in parallel with the other inhibitors. Alkaline sucrose gradient profiles of viral RI pulse labeled under inhibiting conditions (^3H) and cosedimented with RI pulse labeled in the absence of inhibitors (^{32}P) are shown in Fig. 11. The labeling pattern with ara-ATP closely resembles that obtained with ara-CTP (i.e., reduced labeling of long chains and reduced size of short chains). The same features are observed with NEM. Actinomycin D, on the other hand, results in a distribution of label similar to that found in the control pulse. We conclude that actinomycin D does not interfere specifically with the RNA priming or the synthesis of the short chains, but rather exerts some more general inhibitory effect on polyoma DNA synthesis. The similarity of the changes in the pulse-labeling patterns observed with the arabinosyl nucleotides and NEM suggests a common target for these two groups of inhibitors.

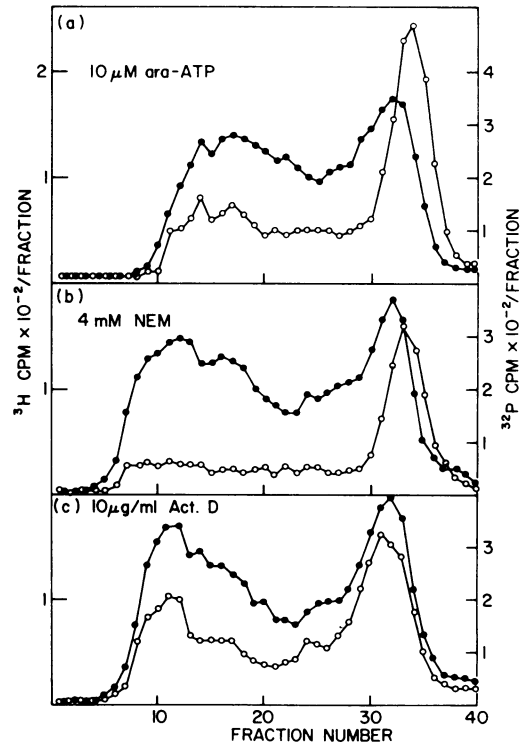

FIG. 11. *Alkaline sucrose gradient sedimentation profiles of viral RI, pulse labeled in the presence of ara-ATP, NEM, and actinomycin D. Incubation mixtures (200 μl; no added dCTP, no added dATP) were pulse labeled at 32 C either with 75 μCi of [α-^{32}P]dCTP (100 Ci/mmol) per ml for 1 min, or with 100 μCi of [^3H]TTP (13.4 Ci/mmol) per ml for 7 min with 10 μM ara-ATP, 4 mM NEM, or 10 μg of actinomycin D per ml present. One-third of the ^{32}P-labeled sample was mixed with each of the ^3H-labeled samples. Purified viral RI was subjected to alkaline sucrose gradient centrifugation as detailed in legend to Fig. 4. (a) [^3H]TTP pulse with 10 μM ara-ATP present, (b) [^3H]TTP pulse with 4 mM NEM present, (c) [^3H]TTP pulse with 10 μg of actinomycin D per ml present. Symbols: O, ^3H counts per minute; ●, ^{32}P counts per minute (control pulse in the absence of inhibitors).*

DISCUSSION

In this communication we report on in vitro DNA synthesis in a crude system derived from polyoma-infected BALB/3T3 cells in the presence of ara-CTP. The discussion will focus on two major aspects of the results, namely (i) the fate of ara-CTP during in vitro DNA synthesis, and (ii) the altered pattern of viral DNA replication in the presence of ara-CTP. The rather specific changes in the replication pattern due to ara-CTP provide some indications as to the number and possible nature of DNA polymerases involved in replication in this system.

(i) Incorporation of ara-CTP during in vitro DNA synthesis. Two mechanisms have been proposed to account for the inhibition of DNA synthesis by ara-CTP. First, ara-CTP might compete directly with dCTP in the polymerizing process irrespective of whether the analogue is incorporated into DNA or not. The second possibility, which is by no means mutually exclusive of the first, is that chain termination is caused by the incorporation of an ara-C residue (27). Our studies on the incorporation of labeled ara-CTP in DNA show that the inhibition by ara-CTP is unlikely to be caused by chain termination since, when ara-CTP was present at concentrations that did not block DNA synthesis completely, the analogue was incorporated internally into the product DNA (Fig. 2) as unmodified ara-C residues. Ara-CTP is incorporated largely at random in the in vitro product, substituting for dCTP to an extent proportional to the ratio of the two nucleoside triphosphates in the reaction mixture (Fig. 2).

Ara-C residues were found to be incorporated to an equal extent into long nascent viral DNA strands and the short chains involved in discontinuous growth. Reversal of the ara-CTP inhibition by dCTP resulted in the joining of ara-C containing short chains to long growing strands (Fig. 5). Therefore, chain termination is not a necessary consequence of the incorporation of ara-C residue. This point is further substantiated by the finding of ara-C containing mature viral DNA (form I, which in its alkali-stable supercoiled structure was indistinguishable from normal form I (Fig. 3).

The reversal of inhibition by dCTP (Fig. 6) indicates a competitive nature of the inhibition by ara-CTP. By using the data in Fig. 1, a K_i for ara-CTP of approximately 3×10^{-6} M can be calculated. This value is in reasonable agreement with the values obtained by Furth and Cohen (12) (1.1×10^{-6} M) and Fox and Goulian (personal communication) (2.2×10^{-6} M) for purified DNA polymerases and by Graham and Whitmore (15) (8.7×10^{-6} M) for crude systems.

(ii) Viral DNA replication in the presence of ara-CTP. The most striking result of analyzing viral replicating DNA (RI) pulse labeled in the presence of 7.5 μM ara-CTP is that there is an increase in the percentage of the pulse label found as short chains when compared to the control. The trivial explanation that this is a consequence of an effectively reduced pulse length due to the decreased synthesis rate can be excluded for the following two reasons. (i) The pulse in the presence of ara-CTP was timed such (5 min) that approximately an equal amount of DNA was made as in the control pulses (1 min). (ii) We have previously shown that even during very short pulses (5 s) at least equal amounts of radioactivity are incorporated into long chains as into short chains (11). This distribution of label between long and short chains during pulses shorter than the lifetime of a single short chain had been interpreted in terms of a semi-discontinuous mode of synthesis in this in vitro system (11). Therefore, a reason for the predominance of label in short chains could be that discontinuous synthesis, which is normally restricted to one side of the growing fork, occurs on both sides during the inhibition by ara-CTP. Such a switch has been demonstrated by Olivera and Bonhoeffer (31) for in vitro *E. coli* DNA replication, where at reduced deoxyribonucleoside triphosphate levels the decreased synthesis rate leads to more frequent initiation of Okazaki fragments. The levels of self-complementarity of isolated short chains (Table 3) do not show higher values for short chains made in the presence of ara-CTP. This suggests that the altered pulse-labeling pattern is not a consequence of a totally discontinuous mode of synthesis in the presence of the inhibitor, but rather that the synthesis of the short chains and the elongation of the long chains are carried out by two different polymerases, the first being relatively resistant and the second being relatively sensitive to partially inhibiting concentrations of ara-CTP. It should be mentioned that high levels of self-complementarity of short chains have been reported for polyoma in vitro in isolated nuclei (32) and for simian virus 40 in vivo (10) and have been interpreted in terms of a totally discontinuous mode of synthesis in these systems.

The other feature of RI pulse labeled in the presence of ara-CTP is the smaller size of the short chains (Fig. 7 and 8, Table 4). The chains synthesized in the presence of ara-CTP are about two-thirds of the size of those made in a control pulse. The size of the regular chains, however, increases slightly during a 30-s chase and, if one uses this larger size as the maximum size for a nascent DNA fragment, then the fragments generated in the presence of ara-CTP are about one-half the normal size. One possible reason for the incomplete synthesis of the short chains is that ara-CTP might interfere with the removal, by an RNase H-like activity, of the RNA primer from the preceding short chain on the parent DNA strand. This would result in a smaller stretch of the template strand being available for copying. Two arguments tend to exclude this as the primary reason for the shorter size of ara-C chains. (i) In the "normal" case, only a small fraction of the chains contain measurable amounts of RNA, and this in turn is maximally 30 bases long (20), an amount that is too small to account for the size difference of short chains being caused by persisting RNA primers. (ii) No increase in the RNA content of short chains synthesized in the presence of ara-CTP was observed (Hunter and Francke, unpublished data). After reversal of the inhibition with excess dCTP, the short chains were found to grow to normal length, indicating that they are separated by gaps. It therefore appears that the synthesis of a full-size short chain is accomplished by three different processes: its priming by a stretch of RNA, extension by a DNA polymerase that is relatively ara-CTP resistant to one-half to two-thirds of its full length, and its completion through gap-filling by a relatively ara-CTP sensitive DNA polymerase. The last step during chain growth, via a discontinuous mechanism, namely the ligasing of short chains to long chains, was found to be

impeded by ara-CTP to an extent not greater than the inhibition of overall DNA synthesis (data not shown), implying that the rate-limiting step in the presence of ara-CTP is the gap-filling required before joining rather than the ligasing itself.

Based on these observations, three different DNA polymerizing processes can be distinguished: (i) continuous elongation of one strand (relatively ara-CTP sensitive), (ii) synthesis of short chains to about two-thirds of their final size (relatively ara-CTP resistant), and (iii) completion of the last one-third of the short chains (relatively ara-CTP sensitive). Other processes, like RNA priming, removal of the primer, and joining of short chains, appear not to be affected, at least not to any greater extent than the overall rate of DNA synthesis. Several DNA-polymerizing activities have been recognized in eukaryotic cells. One of the major activities is of relatively high molecular weight and is isolated from the cytoplasm (2, 7, 37, 29), although it is by no means certain that this reflects its true cellular location. This enzyme shows sensitivity to ara-CTP (13, 28; Fox and Goulian, personal communication) and NEM (28, 37, 39). The second major activity has a nuclear location and a much lower molecular weight (1, 7, 37, 39). This enzyme displays no sensitivity to ara-CTP (13; Fox and Goulian, personal communication) or NEM (37, 39). Since continuous extension and gap-filling are both sensitive to ara-CTP, they might be carried out by the same enzyme. Clearly the cytoplasmic enzyme is a candidate here. This would leave the relatively ara-CTP-insensitive small nuclear polymerase for the synthesis of the short chains. Although this assignment —based on the comparison of the ara-CTP sensitivity of isolated polymerases with the pattern of in vitro DNA replication in the presence of the analogue—appears reasonable, it is in disagreement with conclusions drawn from in vivo experiments with other inhibitors of DNA synthesis.

The accumulation of short DNA fragments has been observed during simian virus 40 DNA replication with fluorodeoxyuridine (34) and with hydroxyurea during polyoma DNA replication (23–25). Laipis and Levine (21) have been able to show directly that the form II component containing short chains, produced during hydroxyurea inhibition, contains physical gaps between the short chains, which can be filled by a combination of the T4 DNA polymerase and the *E. coli* ligase. Both hydroxyurea treatment (36) and fluorodeoxyuridine are known to lower one or more deoxynucleotide triphosphate pools. This reduction would be expected to affect the polymerizing activity with the highest K_m for deoxynucleotide triphosphates to the greatest extent. Since the isolated small nuclear polymerase has a higher K_m for deoxynucleotide triphosphates (35), this enzyme was implicated in gap-filling rather than the synthesis of short chains. This assignment is supported by the finding that, in its purified form, the nuclear enzyme utilizes RNA primers less efficiently than the cytoplasmic one (8). Since the short DNA chains are thought to start with an RNA primer, this observation would argue against the nuclear polymerase accomplishing this process.

At present the difference cannot be resolved. But one reason might be that the properties of isolated enzymes, like sensitivity to inhibitors, K_m for triphosphates, and primer preference, are different when the enzymes are part of a replication complex in the cell. To obtain some direct indications as to which polymerase carries out which step, it would be desirable to study the effect of isolated enzymes on viral DNA replication in vitro. For this reason, we are presently comparing our in vitro system (total cell lysate) to that of isolated nuclei (40), which should contain reduced amounts of cytoplasmic DNA polymerase. If this enzyme plays a role in viral DNA replication, as postulated above, predictable differences of the replication patterns in both systems should be detectable.

ACKNOWLEDGMENTS

We would like to thank Walter Eckhart, in whose laboratory this work was performed, for his interest, advice, and support, and Helen Hesser and Mary Anne Hutchinson for expert technical assistance.

This research was supported by Public Health Service grants CA-13884 and CA-15058 from the National Cancer Institute and contract no. 67-1147 of the Virus Cancer Program of the National Cancer Institute.

LITERATURE CITED

1. Adams, R. L. F., M. A. L. Henderson, W. Wood, and J. G. Lindsay. 1973. Multiple forms of nuclear deoxyribonucleic acid polymerase and their relationship with the soluble enzyme. Biochem. J. **131**:237–246.
2. Bollum, F. J. 1963. Studies on the nature of calf thymus DNA polymerase products. Cold Spring Harbor Symp. Quant. Biol. **28**:21–26.
3. Brink, J. J., and G. A. LePage. 1965. 9-β-D-arabinofuranosyladenine as an inhibitor of metabolism in normal and neoplastic cells. Can. J. Biochem. **43**:1–15.
4. Butel, J. S., and F. Rapp. 1965. The effect of arabinofuranosylcytosine on the growth cycle of simian virus 40. Virology **27**:490–495.
5. Camenier, G. W., and C. G. Smith. 1965. Studies on the enzymatic deamination of cytosine arabinoside. I. Enzyme distribution and species specificity. Biochem. Pharmacol. **14**:1405–1416.
6. Chamberlin, M., and P. Berg. 1964. Mechanism of RNA

polymerase action: formation of DNA-RNA hybrids with single-stranded templates. J. Mol. Biol. **8**:297-313.
7. Chang, L. M. S., and F. J. Bollum. 1971. Low molecular weight deoxyribonucleic acid polymerase in mammalian cells. J. Biol. Chem. **246**:5835-5837.
8. Chang, L. M. S., and F. J. Bollum. 1972. A chemical model for transcriptional initiation of replication. Biochem. Biophys. Res. Commun. **46**:1354-1360.
9. Edgell, M. H., C. A. Hutchison III, and M. Sclair. 1972. Specific endonuclease R fragments of bacteriophage ϕX-174 deoxyribonucleic acid. J. Virol. **9**:574-582.
10. Fareed, G. C., and N. P. Salzman. 1972. Intermediate in SV40 chain growth. Nature (London) New Biol. **283**:277-279.
11. Francke, B., and T. Hunter. 1974. *In vitro* polyoma DNA synthesis: discontinuous chain growth. J. Mol. Biol. **83**:99-121.
12. Furth, J. J., and S. S. Cohen. 1968. Inhibition of mammalian DNA polymerase by the 5'-triphosphate of 1-β-D-arabinofuranosylcytosine and the 5'-triphospl-ate of 9-β-D-arabinofuranosyladenine. Cancer Res. **28**:2061-2067.
13. Gallo, R. C., R. G. Sarlin, S. N. Bobrow, M. G. Sarngadharan, M. S. Reitz, and J. W. Abrell. 1973. RNA directed and primed DNA polymerase activities in tumor viruses and human lymphocytes, p. 251-256. *In* R. D. Wells and R. B. Inman (ed.), DNA synthesis *in vitro*. University Park Press, Baltimore.
14. Graham, F. L., and G. F. Whitmore. 1970. The effect of 1-β-D-arabinofuranosylcytosine on growth, viability, and DNA synthesis of mouse L cells. Cancer Res. **30**:2627-2635.
15. Graham, F. L., and G. F. Whitmore. 1970. Studies in mouse L-cells on the incorporation of 1-β-D-arabinofuranosylcytosine into DNA and on inhibition of DNA polymerase by 1-β-D-arabinofuranosylcytosine 5'-triphosphate. Cancer Res. **30**:2636-2649.
16. Griffin, B. E., M. Fried, and A. Cowie. 1974. Polyoma DNA: a physical map. Proc. Nat. Acad. Sci. U.S.A. **71**:2077-2081.
17. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365-367.
18. Huang, E. S., J. E. Newbold, and J. S. Pagano. 1973. Analysis of simian virus 40 DNA with the restriction enzyme of *Haemophilus aegyptius* endonuclease Z. J. Virol. **11**:508-514.
19. Hunter, T., and B. Francke. 1974. In vitro polyoma DNA synthesis: characterization of a system from infected 3T3 cells. J. Virol. **13**:125-139.
20. Hunter, T., and B. Francke. 1974. *In vitro* polyoma DNA synthesis: involvement of RNA in discontinuous chain growth. J. Mol. Biol. **83**:123-130.
21. Laipis, P. J., and A. J. Levine. 1973. DNA replication in SV40-infected cells. IX. The inhibition of a gap filling step during discontinuous synthesis of SV40 DNA. Virology **56**:580-594.
22. Lark, C., and K. G. Lark. 1964. Evidence for two distinct aspects of the mechanism regulating chromosome replication in *Escherichia coli*. J. Mol. Biol. **10**:120-136.
23. Magnusson, G. 1973. Hydroxyurea-induced accumulation of short fragments during polyoma DNA replication. I. Characterization of fragments. J. Virol. **12**:600-608.
24. Magnusson, G. 1973. Hydroxyurea-induced accumulation of short fragments during polyoma DNA replication. II. Behavior during incubation of isolated nuclei. J. Virol. **12**:609-615.
25. Magnusson, G., V. Pigiet, E. L. Winnacker, R. Abrams, and P. Reichard. 1973. RNA linked short DNA fragments during polyoma replication. Proc. Natl. Acad. Sci. U.S.A. **70**:412-415.
26. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208-217.
27. Momparler, R. 1969. Effect of cytosine arabinoside 5'-triphosphate on mammalian DNA polymerase. Biochem. Biophys. Res. Commun. **34**:465-471.
28. Momparler, R. L., M. Rossi, and A. Labitan. 1973. Partial purification and properties of two forms of deoxyribonucleic acid polymerase from calf thymus. J. Biol. Chem. **248**:285-290.
29. Notari, R. E., M. L. Chin, and A. Cardoni. 1970. Intermolecular and intramolecular catalysis in deamination of cytosine nucleosides. J. Pharm. Sci. **59**:28-32.
30. Okazaki, R., M. Arisawa, and A. Sugino. 1971. Slow joining of newly replicated DNA chains in DNA polymerase I-deficient *Escherichia coli* mutants. Proc. Natl. Acad. Sci. U.S.A. **68**:2954-2957.
31. Olivera, B. M., and F. Bonhoeffer. 1972. Discontinuous DNA replication *in vitro*. I. Two distinct size classes of intermediates. Nature (London) New Biol. **240**:233-235.
32. Pigiet, V., E. L. Winnacker, R. Eliasson, and P. Reichard. 1973. Discontinuous elongation of both strands at the replication forks in polyoma DNA replication. Nature (London) New Biol. **245**:203-205.
33. RamaReddy, G. V., M. Goulian, and S. S. Hendler. 1971. Inhibition of *E. coli* DNA polymerase II by ara-CTP. Nature (London) New Biol. **234**:286-288.
34. Salzman, N. P., and M. M. Thoren. 1973. Inhibition of the joining of DNA intermediates to growing simian virus 40 chains. J. Virol. **11**:721-729.
35. Sedwich, W. D., T. S. F. Wang, and D. Korn. 1972. Purification and properties of nuclear and cytoplasmic deoxyribonucleic acid polymerases from human KB cells. J. Biol. Chem. **247**:5026-5033.
36. Skoog, L., and B. Nordenskjöld. 1971. Effects of hydroxyurea and 1-β-D-arabinofuranosyl-cytosine on deoxyribonucleotide pools in mouse embryo cells. Eur. J. Biochem. **19**:81-89.
37. Smith, R. G., and R. C. Gallo. 1972. DNA dependent DNA polymerases I and II from normal human blood lymphocytes. Proc. Natl. Acad. Sci. U.S.A. **69**:2879-2884.
38. Sutton, W. D. 1971. A crude nuclease preparation suitable for use in DNA reassociation experiments. Biochim. Biophys. Acta **240**:522-531.
39. Weissbach, A., A. Schlabach, B. Fridlender, and A. Bolden. 1971. DNA polymerase from human cells. Nature (London) New Biol. **231**:167-170.
40. Winnacker, E. L., G. Magnusson, and P. Reichard. 1972. Replication of polyoma DNA in isolated nuclei. I. Characterization of the system from mouse fibroblast 3T6 cells. J. Mol. Biol. **72**:528-537.