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In Vitro Polyoma DNA Synthesis: Requirement for Cytoplasmic Factors

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Purified nuclei from polyoma-infected mouse (3T3) cells were found to be greatly reduced in their ability to synthesize viral DNA in vitro when compared with a crude system consisting of an unfractionated hypotonic lysate of the infected cells. The synthetic capacity of the nuclei could be fully reconstituted when a high-speed cytoplasmic supernatant was added back to them. Cytosols from uninfected mouse, monkey, and hamster cells were equally as effective in stimulating purified nuclei as that of virus-infected mouse cells. Optimal complementation required high concentrations of the cytosol, and most of the complementing activity was destroyed by heating to 60 C. Dialysis had no effect on the activity. Analysis of the viral DNA synthesized in purified nuclei showed an accumulation of Okazaki-type short DNA chains, which could be chased into viral progeny DNA strands if cytosol was added back to the nuclei. Kinetic analysis of the pulse-labeling pattern of viral replicative DNA showed a strong dependence of the extension of viral progeny strands and of the processing of Okazaki-type fragments on the amount of cytosol present during the reaction. It is suggested that the cytoplasmic DNA polymerase might be one of the active components in the cytosol, but most likely not the only one.

In vitro systems for DNA synthesis, derived from cells infected with the papovavirus polyoma, have extensively been used as model systems for the biochemical analysis of mammalian DNA replication. Like the simian papovavirus SV40 (21), the murine virus polyoma depends largely on host cell functions for the synthesis of the viral DNA. After the onset of viral DNA replication, only one viral gene is required for continued replication. Experiments both in vivo (5) and in vitro (7) have demonstrated that this viral function is responsible for the initiation of each round of DNA synthesis. After this initiation event, the synthesis of the progeny strands in viral replicative intermediate (RI) and the generation of mature double-stranded circular DNA (form I) appear to be accomplished by cellular enzymes. The in vitro systems developed so far have been found to be defective in the initiation of new rounds of replication but are able to synthesize viral DNA in a semiconservative fashion and to generate mature viral form I DNA molecules utilizing viral RI present in the cells at the time of preparation of the system (13, 21). Analyzing the course of DNA replication in such systems, strand elongation was shown to proceed, at least in part, in a discontinuous manner, involving short Okazaki-type DNA pieces as intermediates, which are initiated by an RNA primer (6, 14, 16). Minor differences in the processing of short DNA chains in isolated nuclei (16) and that in an unfractionated lysate of the infected cells (6) had indicated that cytoplasmic components might play a role in DNA synthesis. The requirement for cytoplasmic factors for optimal in vitro DNA synthesis has been established in a variety of other systems, where chromosomal rather than viral DNA was investigated (2, 8, 11). The disadvantage of such systems is that the size of chromosomal DNA makes the isolation and characterization of the in vitro product rather difficult, and for that reason no information is available yet as to whether the cytoplasmic factor(s) is required for any specific steps during DNA replication. Using the unfractionated crude lysate from polyoma-infected mouse cells, for which the mechanism of replication has been studied in detail (13), and comparing it with the synthesis in purified nuclei, we were able to show that DNA synthesis exhibits a strong dependence on the presence of cytosol. By analysis of the in vitro viral DNA product in the absence and presence of cytosol,

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we have been able to draw rather specific conclusions about the nature and function of the cytoplasmic factor(s).

MATERIALS AND METHODS

Virus, cells, and growth conditions. The polyoma ts1260 mutant was used in all experiments. It belongs to a late complementation group and behaves like wild type with respect to viral DNA replication.

BALB/3T3 cells (mouse fibroblasts) were obtained from S. Aaronson, and BSC-1 (monkey) and BHK (baby hamster kidney) cells were obtained from M. Stoker. All cells were grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum. BALB/3T3 cells, 4 days after reaching confluency and without fluid changing, were used as resting cells and before reaching confluency (80% of the culture dish covered) as growing cells. Serum stimulation was performed 1 day after reaching confluency by fluid changing with fresh medium containing 10% calf serum and used 24 h later (all procedures at 37 C).

Infected BALB/3T3 cells were used 36 h after infection with polyoma ts1260 at 32 C (multiplicity of infection, 20 PFU/cell).

Preparation of lysate, nuclei, and cytosol. Hypotonic buffer consisted of: 20 mM *N*-2-hydroxyethyl piperazine-*N'*-2'-ethane sulfonic acid (HEPES) (pH 7.8), 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 1 mM dithiothreitol. Sucrose buffer contained 2.5 M sucrose, 200 mM HEPES, and 500 mM KCl; isotonic buffer was a 9:1 mixture of hypotonic and sucrose buffers.

Lysates were prepared as described previously (13) by treatment of the infected cells with hypotonic buffer on the dish and mixing 9 parts of lysate with 1 part of sucrose buffer.

Nuclei were prepared from the hypotonic lysate (before the addition of sucrose buffer) by diluting the lysate fourfold with hypotonic buffer and bringing it to 0.1% with Nonidet P-40. The mixture was then layered over 20% (wt/vol) Ficoll in isotonic buffer and centrifuged for 15 min at 15,000 $\times$ *g* in a conical centrifuge tube (V. Pigiet, personal communication). For quantitative recovery of the nuclei, not more than the equivalent of 1.5×10^7 cells was centrifuged in one tube of 2.5-cm diameter. The pelleted nuclei were washed twice in 40 volumes of isotonic buffer and finally resuspended in isotonic buffer of a volume corresponding to 1.1 times that of the original hypotonic lysate. For complementation studies with cytosol, the nuclei were directly resuspended in cytosol after the second wash.

Cytosol was prepared by homogenizing the hypotonic lysate with 12 strokes in a Dounce homogenizer. The homogenate was made isotonic with 0.1-volume sucrose buffer and centrifuged at 30,000 $\times$ *g* for 2 h at 2 C. The supernatant was used as the cytosol fraction. Dialysis was against two 3-h changes of 500 volumes of isotonic buffer.

All procedures were carried out at 4 C.

In vitro incubation conditions. All in vitro incubation conditions contained the following additions (final concentrations), including the components of the isotonic buffer: 1 mM ATP; 100 μM CTP, GTP, UTP, dATP, dCTP, and dGTP; 15 μM TTP; 5 mM creatine phosphate and creatine phosphokinase; 1 mM dithiothreitol; 1 mM EDTA; 40 mM HEPES, pH 7.8; 100 mM KCl; 5 mM $MgCl_2$; 1 mM $MnCl_2$; 0.5 mM $CaCl_2$; and 50 μCi of [^{3}H]TTP/ml (54 Ci/mmol). Deviations from these incubation conditions will be mentioned with individual experiments. All incubations were at 32 C. Termination by a modified Hirt (12) extraction and determination of incorporated radioactivity were as described previously (13).

Determination of TTP pools. TTP pools in the lysate, purified nuclei, and the reconstituted system were determined by incubating parallel samples for 10 min. Ten different concentrations of added unlabeled TTP (between 10 and 100 μM) were used in the presence of a constant amount of labeled [^{3}H]TTP. Each concentration was assayed in triplicate. Calculations of the pool size were as described previously (13).

Analysis of viral DNA. For the characterization of viral DNA, the Hirt supernatant fraction was routinely subjected to preparative sedimentation through a neutral sucrose gradient from which the fractions containing viral DNA were recovered. After concentration by ethanol precipitation and extraction with phenol-chloroform, the DNA was further analyzed by sedimentation through alkaline sucrose gradients at 55 K in an SW56 rotor of a Beckman L2-65 ultracentrifuge. For the separation of form I from denaturable DNA, gradients were spun for 90 min, and for the separation of long and short progeny strands, they were spun for 5 h. For experimental details, see Hunter and Francke (13).

Chemicals and radioisotopes. The source and specific activities of α-^{32}P- or ^{3}H-labeled deoxynucleoside triphosphates were as described previously (13). Ficoll was purchased from Pharmacia, Upsala, Sweden.

RESULTS

Figure 1 shows the time courses of incorporation of [^{3}H]TTP into acid-insoluble material for viral DNA (Hirt supernatant, Fig. 1a) and cellular DNA (Hirt pellet, Fig. 1b). Both the initial rate and the overall level of synthesis of DNA in purified nuclei (Materials and Methods) were reduced to about one-tenth of the values found for the complete lysate. When purified nuclei were complemented with the cytosol fraction of the same cells, both the initial rate and the overall level of viral and cellular DNA synthesis were restored to levels almost identical to those found in the unfractionated lysate. Several trivial reasons for the restricted synthesis in purified nuclei and the efficient complementation by cytosol can be ruled out.

(i) We verified that the concentration of purified nuclei in the reaction mixture was the same as in the unfractionated lysate by prela-

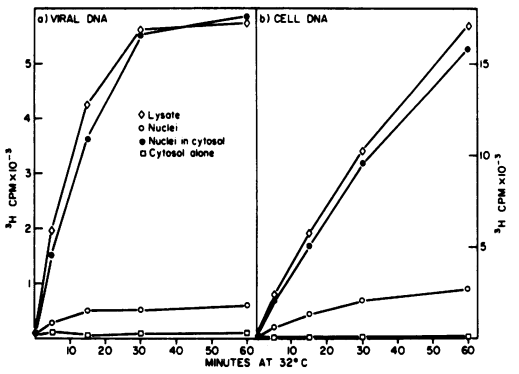

FIG. 1. *Time courses of [^{3}H]TTP incorporation in the unfractionated lysate, purified nuclei, and the reconstituted system. Lysate, nuclei, and cytosol were prepared from ts1260-infected 3T3 cells. Five 50-μliter incubations were prepared for each system and incubated at 32 C. After the times indicated, the incubations were terminated and separated into the Hirt supernatant fractions (viral DNA [a]) and Hirt pellet (cellular DNA [b]).*

beling the infected cells in vivo with [^{3}H]deoxythymidine for 12 h (labeling predominantly form I DNA) or for 2 min (labeling predominantly viral RI). We recovered an identical amount of radioactivity in form I DNA and viral RI, respectively, from the lysate and from purified nuclei resuspended for incubation in the same volume as the volume of lysate from which they were derived (data not shown). We conclude that the limited synthesis in purified nuclei is not due to a reduction in the amount of template (viral RI) available.

(ii) The complementing activity in the cytosol is not a result of possible additional templates present in the cytosol fraction, since incubation of the cytosol alone under the same conditions did not result in significant incorporation of [^{3}H]TTP (Fig. 1).

(iii) To test whether the template for viral DNA synthesis might possibly be degraded during the in vitro reaction, thus limiting the extent of in vitro synthesis, in vivo prelabel experiments described in (i) were analyzed by sedimenting the viral DNA under alkaline denaturing conditions. Long-term prelabeled form I DNA (83% of the radioactivity in the Hirt supernatant) was found to remain completely alkali stable during a 60-min in vitro incubation in purified nuclei, and no shift toward smaller strand sizes was observed for short-term prelabeled viral RI (compare Fig. 3). Previous experiments with nuclei isolated by different methods had routinely shown extensive degradation of prelabeled DNA (Hunter and Francke, unpublished observations). The present method—particularly the sedimentation step through Ficoll—avoids this complication. The reason for the effectiveness of the Ficoll step is not known. A possible explanation might be a more complete removal of lysosomal nucleases that were liberated during the fractionation of the lysate.

(iv) To rule out an effect of the ionic constitution of the cytosol, the optima for Mg^{2+}, Mn^{2+}, and K^+ for synthesis in purified nuclei were determined and found to be similar to those of the complete lysate (data not shown). In addition, dialysis of the cytosol against the same buffer used for washing and incubating the nuclei did not decrease its complementing activity (see below). The stimulation of cytosol is therefore not a result of a dialyzable low-molecular-weight compound.

(v) Incorporation of [^{3}H]TTP in purified nuclei was found to be strongly dependent on the presence of the other three deoxynucleoside triphosphates. As determined in two independent preparations, the pool of TTP in purified nuclei is three- to fivefold lower than in the lysate (Table 1), and most of the TTP appears to be present in the cytosol and is not easily dialyzable (13). To exclude a suboptimal TTP concentration as a possible reason for the limited synthesis in purified nuclei, the minimal concentration of added unlabeled TTP for maximal rate and extent of synthesis was determined and found to be 10 μM. Therefore, the reaction mixture for all in vitro incubations presented here contained 15 μM unlabeled TTP. Calculating the specific activities for TTP based on the pool sizes presented in Table 1, the stimulation of cytosol on actual amounts of DNA synthesized in purified nuclei is about 15-fold.

The cytosol fraction used in the experiment reported in Fig. 1 was prepared from the same

TABLE 1. *TTP pools in the unfractionated lysate, in isolated nuclei, and in the reconstituted system*

System	TTP pools (μM)		
	Expt 1		Expt 2 (viral DNA)
	Viral DNA	Cellular DNA	
Lysate	10	10	11.5
Nuclei	3	2	1.5
Nuclei in cytosol	10	8	8.5

[a] Experiments 1 and 2 represent two independent determinations performed on two different sets of infected cells. In experiment 2 only the viral DNA fraction was analyzed.

virus-infected cells used to isolate the nuclei. When cytosol from mock-infected cells (i.e., provided with fresh medium and 10% serum 36 h before use) was tested, its complementing ability was found to be identical. Therefore, all subsequent experiments were done with cytosol from uninfected cells (see also Table 3).

An analysis of the viral DNA synthesized in vitro in a 60-min incubation is presented in Fig. 2. Sedimentation profiles in neutral sucrose gradients are shown of Hirt supernatants after an in vivo pulse prelabel with [^{3}H]deoxythymidine (Fig. 2a) and after in vitro incubation in the presence of [α-^{32}P]TTP in the unfractionated lysate (Fig. 2b), purified nuclei (Fig. 2c), and in the reconstituted system (Fig. 2d). The fractions containing viral DNA (6–18) were recovered, concentrated, and sedimented through alkaline sucrose gradients to separate rapidly sedimenting form I from the remaining denaturable form II and RI (Table 2). Practically no mature form I DNA is formed in purified nuclei, whereas the conversion of the prelabeled RI to form I is rather efficient in the lysate (57%) and in nuclei in the presence of cytosol from uninfected cells (49%). This observation demonstrates clearly that no diffusible viral function is required for any step leading to mature form I DNA. Despite the low amount of in vitro incorporation into viral DNA in nuclei alone, the sedimentation profile of the pulse prelabel in the neutral gradient (Fig. 2a) changed (Fig. 2c). There was a broad spectrum ranging from some remaining 25*S* material (RI) to a predominance of 16*S* (form II). This could reflect a change in tertiary structure (e.g., reduction in the number of superhelical turns) or a limited number of breaks in the template strands. But since no material smaller than 16*S* was formed, it was not likely to be caused by extensive degradation. To characterize the progeny strands further, the viral DNA, after the in vivo pulse and subsequent in vitro incubation of purified nuclei, was sedimented through an alkaline gradient to resolve the size of the single strands up to 16*S* (full-size linear viral DNA) (Fig. 3). There was some increase in the average size of the prelabeled DNA, and no major degradation of the ^{3}H-labeled progeny strands took place during the in vitro incubation, confirming the statement made in (iii) above. The in vitro label in the experiment (^{32}P in Fig. 3b) showed a prominent peak around 5*S*, the sedimentation value of Okazaki-type short DNA chains involved as intermediates in discontinuous chain growth (6). Similar findings have been reported for nuclei from SV40-infected monkey cells (17). This suggests that purified nuclei are capable of synthesizing short DNA chains but in the absence of cytoplasm are unable to process them efficiently to form longer chains.

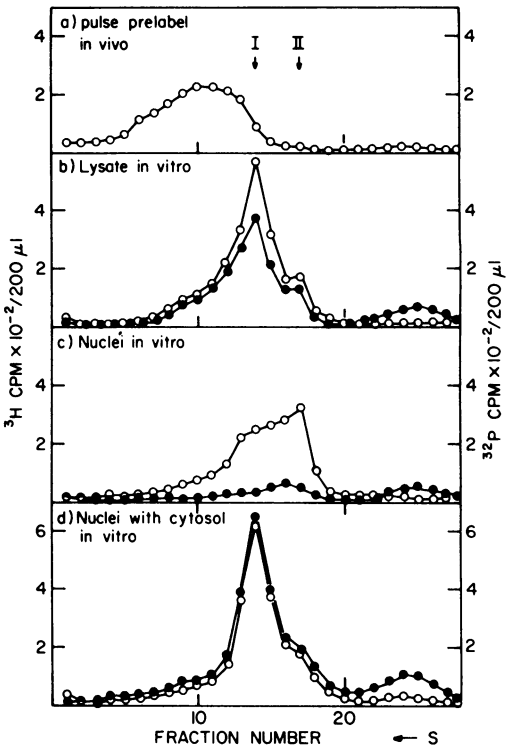

FIG. 2. *Preparative neutral sucrose sedimentation of viral DNA, pulse prelabeled in vivo ([^{3}H]deoxythymidine) and incubated in vitro for 60 min ([α-^{32}P]TTP). The ts1260-infected 3T3 cells were pulse labeled in vivo with 200 µCi of [^{3}H]deoxythymidine per ml for 5 min at 32 C, and a hypotonic lysate was prepared immediately after the pulse. The lysate was divided into four equal parts; one part was subjected to Hirt extraction (a), one was made isotonic (b), and the nuclei were purified from the remaining two parts. Half the nuclei were resuspended in isotonic buffer (c) and the other half in cytosol from uninfected serum-stimulated 3T3 cells (d). In vitro incubation was in 200-µliter samples under the same conditions as in Fig. 1, except that [α-^{32}P]TTP (100 µCi/ml, 108 Ci/mmol) was substituted for [^{3}H]TTP, for 60 min at 32 C. The Hirt supernatant fractions were sedimented through neutral sucrose gradients. 200 µliters of each of the 1.2-ml fractions was sampled for characterization of incorporated radioactivity. The top of the gradient corresponds to the right of the diagram. Symbols: (○) ^{3}H counts per minute, pulse prelabel; (●) ^{32}P counts per minute in vitro label. I, II, Positions of viral DNA I and II of a ^{32}P-labeled marker added to the gradient in (a).*

TABLE 2. *Characterization of viral DNA, pulse prelabeled in vivo (^3H) and incubated in vitro for 60 min (^{32}P)*[a]

Viral DNA	Total counts/min in viral DNA	% of total viral DNA in:	
		Alkali-stable form I	Alkali-denaturable form II and RI
In vivo pulse	^3H, 1,706	4	96
In vitro incubation:	^3H, 1,845	57	43
Lysate	^{32}P, 7,076	51	49
Isolated nuclei	^3H, 1,728	5	95
	^{32}P, 2,255	0.8	99.2
Nuclei in cytosol	^3H, 1,636	49	51
	^{32}P, 10,701	38	62

[a] Fractions 6 through 18 of the neutral gradients presented in Fig. 2 were recovered and half of each sample was analyzed by alkaline sucrose gradient sedimentation for 1.5 h. Total counts per minute is the sum of all radioactivity recovered from each gradient. Form I is the radioactivity sedimenting at 53S, and form II + RI is that sedimenting at ≤ 18S.

To test this possibility, a series of pulses was performed in the unfractionated lysate, purified nuclei, and in nuclei with different concentrations of cytosol present (Fig. 4). The data presented in this figure were obtained by sedimenting viral RI, pulse labeled with [α-^{32}P]TTP (a) or [^{3}H]TTP (b–d) for the times indicated, through alkaline sucrose gradients and integrating the radioactivity present in short (2 to 8S) and long chains (8 to 16S) separately. This type of analysis has been discussed in detail previously (6). The label accumulating in long chains represents two processes: (i) the continuous extension of the growing strand on one side of the replication fork, and (ii) the joining of short chains to the growing strand on the other side. The accumulation of radioactivity in short chains leveled off as soon as the synthesis of short chains and their joining reached equilibrium (Fig. 4A) (6). Purified nuclei in cytosol showed essentially similar kinetics, except that all processes occurred at a slower rate (Fig. 4B). Dilution of the cytosol or leaving it out completely resulted in a relative reduction of radioactivity in long chains (Fig. 4C, D), indicating a reduced rate of elongation of the continuously growing strand, but appeared to affect the synthesis of short chains to a lesser degree. In Fig. 5, three of the alkaline gradients used to obtain the data shown in Fig. 4 are presented. They show the pattern of RI labeled in purified nuclei for 1 min (a) and 4 min (b) and labeled in nuclei in the presence of undiluted cytosol for 1 min (c). All gradients contained RI labeled in the unfractionated lysate for 1 min with [α-^{32}P]TTP as a reference. Short chains, labeled in nuclei, appeared to be noticeably smaller than in the complete system, even after a 4-min label. This defect can be overcome by the cytosol fraction. This observation, in combination with the fact that even the viral DNA labeled in nuclei alone for 60 min (Fig. 3b) contained a large amount of unjoined short chains, suggests that the cytosol is also required for the gap-filling step between the short chains, thought to be necessary before their joining (15, 18; Hunter and Francke, submitted for publication). Label, which accumulated in short chains in purified nuclei, could be chased into long chains if cytoplasm was added back to the incubation (Fig. 6). A 20-min pulse label with [^{3}H]TTP in nuclei (a) was followed by a 20-min chase with excess TTP either in the absence (b) or presence (c) of cytosol. DNA synthesized during the chase was labeled with [α-^{32}P]dCTP. In the absence of cytosol, the sedimentation pattern in alkaline

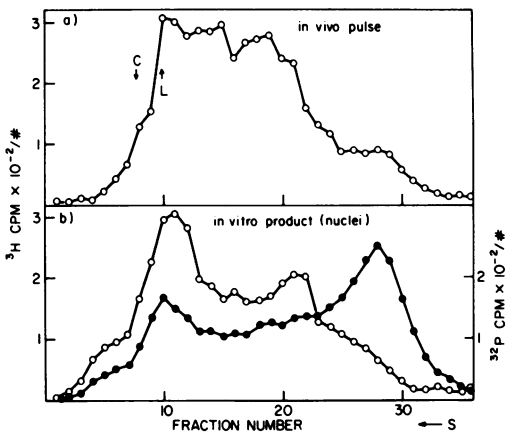

FIG. 3. *Alkaline sucrose gradient sedimentation of viral DNA, pulse prelabeled in vivo (a) ([^{3}H]deoxythymidine) and incubated in vitro for 60 min in purified nuclei (b) ([^{32}P]TTP). Fractions 6 through 18 from the neutral gradients shown in Fig. 2a and c were recovered, and half of the DNA sedimented through alkaline sucrose gradients for 5 h. Total fractions were assayed for radioactivity. Sedimentation was from right to left. Symbols: (○) ^{3}H counts per minute, pulse prelabel; (●) ^{32}P counts per min, in vitro label. C, L, Positions of circular (18S) and linear (16S) single strands of viral form II DNA (^{32}P labeled) present in the first gradient.*

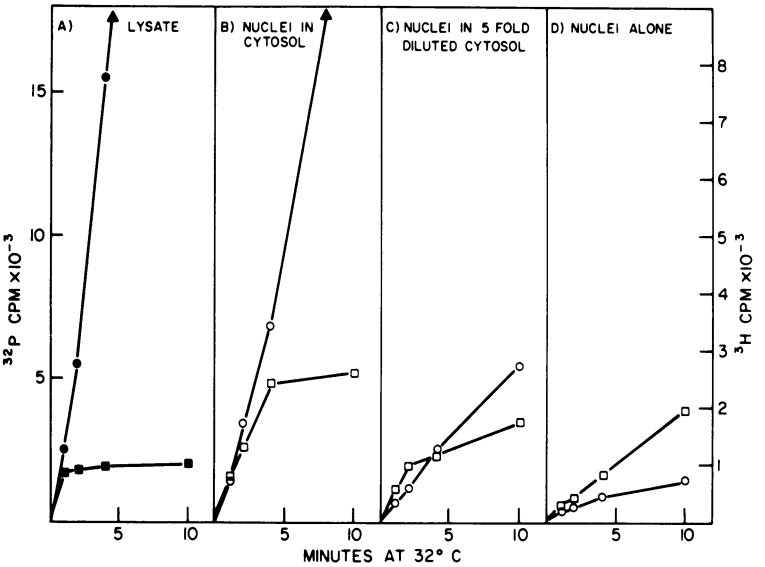

FIG. 4. *Pulse labeling kinetics of short and long DNA chains in viral RI. 5 ml of hypotonic lysate, obtained from ts1260-infected 3T3 cells, was divided into five parts. Two parts were made isotonic, and one was pulse labeled with [α-^{32}P]TTP (500 μCi/ml) for 1 min. The Hirt supernatant from this pulse was divided into 12 parts and added to each of the ^{3}H-labeled samples (compare Fig. 5). The other 1 ml of lysate was pulse labeled in four 250-μliter portions for 1, 2, 4, and 10 min with [α-^{32}P]TTP (200 μCi/ml) (A). Nuclei purified from the remaining 3 ml were resuspended in cytosol from uninfected, serum-stimulated 3T3 cells (B), in the same cytosol diluted 1:5 with isotonic buffer (C), and in isotonic buffer alone (D). Labeling was for 1, 2, 4, and 10 min with [^{3}H]TTP (500 μCi/ml). All pulses were started at 1 min after the beginning of the in vitro incubation. Viral RI, purified by preparative neutral sucrose gradient sedimentation, was subjected to alkaline sucrose gradient sedimentation, and the radioactivity in short and long chains was determined by adding the counts per minute of the respective areas of the gradients. Symbols: (□) Short chains (2 to 8S); (○) long chains (9 to 16S); (closed symbols) ^{32}P; (open symbols) ^{3}H.*

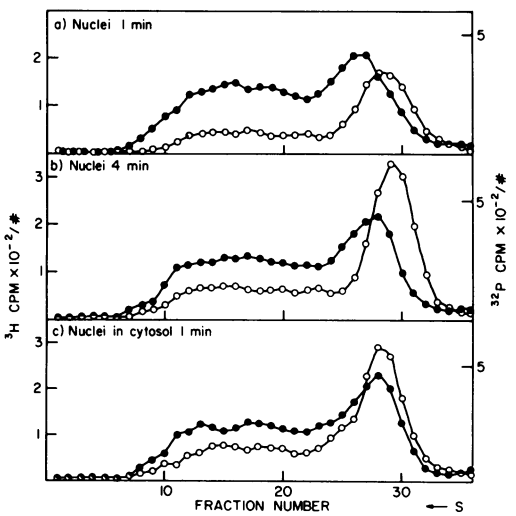

FIG. 5. *Alkaline sucrose gradient sedimentation patterns of selected samples of the experiment presented in Fig. 4. Shown are the 1-min (a) and 4-min (b) pulses in nuclei without, and the 1-min pulse (c) in nuclei with, concentrated cytosol. Centrifugation was as described in Fig. 3. Symbols: (○) ^{3}H counts per minute; (●) ^{32}P counts per minute, profile of the reference pulse for 1 min in the unfractionated lysate.*

sucrose of the ^{3}H pulse remained unchanged during the chase. In addition, the ^{32}P profile indicates that the pattern of DNA synthesis did not change either during this time. With cytosol present, practically all ^{3}H-labeled short chains were joined into long chains, and the ^{32}P profile shows a pattern expected for a 20-min label under normal synthesis conditions, i.e., no evidence for accumulation of short chains. The total ^{3}H counts per minute recovered from viral RI in the three samples were: 744 (a), 712 (b), and 745 (c). The ^{32}P radioactivity in Fig. 6c and d cannot be compared directly since the dCTP pools in purified nuclei and in the cytosol are not known. However, since the dCTP pool in the unfractionated lysate is the highest of all four deoxyribonucleotides (13), the specific activity of the [α-^{32}P]dCTP was considerably lower during the chase in the presence of cytosol than in its absence. This experiment shows that the accumulation of the shorter chains in purified nuclei during incubation is not a consequence of irreversible damage to the system, since they can be processed and joined normally if cytosol is added back.

The series of experiments presented in Table 3 was carried out to determine what influence

the source of the cytosol (cell type and culture conditions) and its concentration had on the stimulation of DNA synthesis in isolated nuclei. Bernard and Brent (2) have reported that the stimulation of DNA synthesis in isolated HeLa cell nuclei varied with the cell cycle, being optimal with cytosol from S-phase cells. Since unsynchronized cells were used in experiment 1 (Table 3), no large differences in the stimulation by the three different cytosol preparations was seen, but it is apparent that resting cells are slightly less and freshly serum-stimulated cells more efficient than growing cells. This is reflected both in the synthesis rate and in the yield of mature form I. All experiments so far were performed with mouse cells, which are the

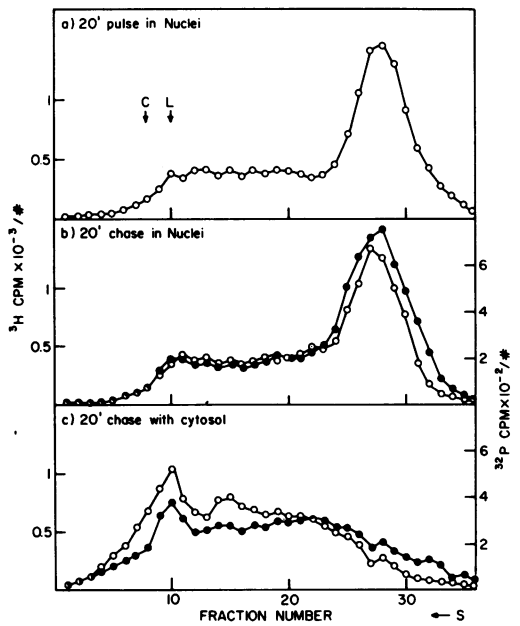

FIG. 6. *Chase of a 20-min pulse label of viral RI in purified nuclei (a) in the absence (b) and presence (c) of cytosol. Purified nuclei from ts1260-infected 3T3 cells were resuspended in isotonic buffer at twice the usual concentration (600 μliters). 200 μliters each of isotonic buffer and of cytosol from uninfected cells was prepared for the chase. All three samples contained the additions for in vitro DNA synthesis as described in Materials and Methods, except the dCTP concentration was reduced to 15 μM. The two chase mixtures contained in addition 300 μM TTP and 50 μCi of [α-^{32}P]dCTP per ml (96 Ci/mmol). The nuclei were pulse labeled with [^{3}H]TTP (100 μCi/ml) for 20 min; 200 μliters was extracted immediately (a), and 200 μliters each was chased for 20 min with the chase mixture in buffer (b) or in cytosol (c). Alkaline sucrose gradient sedimentation profiles of purified viral RI are shown. Symbols:* (○) *^{3}H counts per minute (pulse);* (●) *^{32}P counts per minute (chase). (C, L) See legend to Fig. 3.*

TABLE 3. *DNA synthesis in isolated nuclei in the presence of various cytosols*[a]

Expt no.	DNA synthesis rate (5-min incorporation)		60-Min viral DNA product	
	Viral DNA	Cell DNA	Total counts/min	Alkali-stable form I (%)
1. Nuclei alone	436	707	1,168	0.5
Nuclei in cytosol from:				
Serum-stimulated 3T3 cells	1,108	2,817	4,054	52
Resting 3T3 cells	928	2,051	3,205	41
Growing 3T3 cells	957	2,223	3,832	45
2. Nuclei alone	350	850	910	0.3
Nuclei in cytosol from:				
3T3 cells	945	2,150	3,470	30
BHK cells	870	2,205	2,880	26
BSC-1 cells	1,210	2,345	4,530	29
3. Nuclei alone	350	850	910	0.3
Nuclei in cytosol from 3T3 cells:				
Concentrated cytosol	930	2,350	3,435	31
Cytosol diluted 1:2	1,225	2,185	4,425	25
1:5	805	1,520	3,010	
1:10	680	1,250	2,780	2
1:20	605	1,210	2,220	
1:50	575	1,015	1,890	0.5
1:100	410	925	1,270	

[a] For each experiment, a [^{3}H]TTP incorporation time course was performed as described in the legend to Fig. 1. The data for viral and cellular DNA synthesis rates and for the total counts per minute in the 60-min viral DNA product are taken from this time course. In addition, a 200-μliter sample was incubated for 60 min and analyzed as outlined in the legend to Fig. 2 and Table 2 to calculate the percentage of form I synthesized. In experiment 3 the dilutions were made with the indicated volumes of isotonic buffer. The total number of nuclei was the same in each series.

permissive host for the polyoma lytic cycle. Cytosol from hamster cells (BHK) and monkey cells (BSC-1) were about equally efficient (percentage of mature form I synthesized; Table 3, experiment 2). The slight apparent variation in the efficiency of [^{3}H]TTP incorporation may not be due to inherent differences but possibly to variation in the pool size of TTP between different cell lines (not determined). This result demonstrates that the factors for permissiveness for polyoma growth are not involved in the continuation and completion of initiated rounds of DNA replication. It appears, rather, that the stimulatory cytoplasmic factor(s) is somewhat unspecific and may be common to all mammalian cells. The stimulation by cytosol from mouse 3T3 cells (experiment 3, Table 3) showed a strong concentration dependence, indicating that for optimal synthesis (as measured by the yield of form I) a high concentration of the

cytoplasmic factor(s) is required. The method used to prepare the cytosol (high-speed centrifugation of a lysate after Dounce homogenization) does not recover all the cytoplasm quantitatively from the lysate, since pieces of cytoplasm sticking to the nuclei and other organelles and membranes will be pelleted. Since, in addition, the components of the lysate are already diluted by a factor of about two compared with intact cells (13), the "concentrated" cytosol represents a considerable dilution compared with the in vivo situation. This circumstance may well be the reason for the extended time scale in the pulse-labeling kinetics shown in Fig. 4b. The increase in absolute radioactivity incorporated with the 1:2-diluted cytosol (experiment 3, Table 3) was probably a result of the increased specific activity of [^{3}H]TTP due to the dilution of the TTP pool. A concentration dependence of DNA synthesis could also be demonstrated in the unfractionated lysate (Table 4), although to a lesser degree. However, since the lysate consists primarily of clumps of nuclei embedded in cytoplasm (13), dilution will probably not be a very effective way of decreasing cytoplasmic nuclear interaction.

Preliminary characterization of the stimulating activity indicates that it is most likely to be a protein fraction of the cytosol. It is nondialyzable and exposure to 60 C for 5 min destroys most of the complementing activity (Fig. 7), as does treatment with trypsin (data not shown). It is not affected by preincubation at 32 C for up to 15 min. The residual activity after heating at 60 C is very reproducible and is still observed after heating for 60 min. The heat-stable component(s) of the cytosol also appears to be nondialyzable, but no indications as to its nature have been obtained so far. The cytosol fractions after the various treatments described in Fig. 7 were also tested for their influence on the pulse-labeling pattern of viral DNA (Table 5). The typical pattern seen in nuclei alone (44% of the label still in short chains) was essentially unchanged by the presence of heated (60 C) cytosol, indicating that the heat-stable component alone cannot overcome the deficiency, whereas dialysis or preincubation at 32 C has no influence on the cytosol's capacity to restore the normal synthesis pattern (i.e., most of the label is found in long chains after a 20-min pulse).

TABLE 4. *Concentration dependence of in vitro DNA synthesis in the unfractionated lysate*[a]

System	DNA synthesis rate (5-min incorporation)		60-Min viral DNA product	
	Viral DNA	Cell DNA	Total counts/min	Alkali-stable form I (%)
Concentrated lysate	1,107	2,467	4,544	48
Lysate diluted 1:2	886	1,805	3,421	45
1:5	828	1,168	2,787	35
1:10	735	1,072	2,625	32

[a] Experimental details were as outlined in legend to Fig. 3, experiment 3. The unfractionated lysate from ts1260-infected 3T3 cells was incubated in 50-μliter portions (concentrated lysate) and diluted with isotonic buffer of the volumes indicated (diluted lysates).

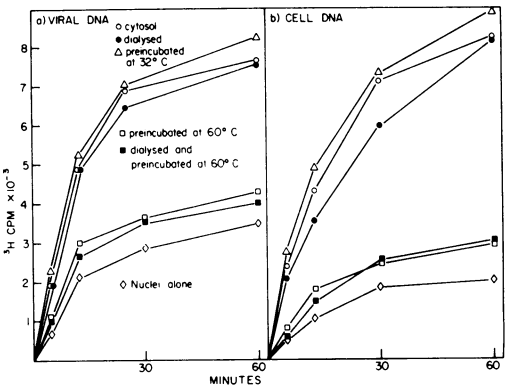

FIG. 7. *Influence of dialysis and heat treatment of cytosol on [^{3}H]TTP incorporation in purified nuclei into viral (a) and cellular (b) DNA. Experimental details were as described in legend for Fig. 1, except that 100-μliter incubations were used and the cytosol was prepared from uninfected cells.*

DISCUSSION

In vitro DNA synthesis in purified nuclei from polyoma-infected mouse fibroblasts (3T3 cells) was found to be greatly restricted when compared with a complete system consisting of unfractionated hypotonically lysed cells. The synthetic capacity of the nuclei (including the conversion of viral RI into form I) could be fully reconstituted by the addition of cytosol with synthesis rates 50 to 70% of the unfractionated lysate. Heat lability of the complementing activity and its nondialyzability indicate that it is a protein (or several proteins) of the cytosol. These observations resemble those made for chromosomal DNA synthesis in HeLa cells (2, 8, 11). A heat-stable cytoplasmic fraction has been described by Shimida and Terayama (20) for infant rat brain nuclei. The nature of the small but reproducible stimulation with heat-inactivated cytosol reported here remains obscure,

TABLE 5. *Influence of cytosol on the pulse-labeling pattern of viral RI*[a]

20-Min label with [^{3}H]TTP in isolated nuclei with:	Total counts/min in viral RI	Count/min (%) in:	
		Long chains	5S DNA
No cytosol	2,540	56	44
Untreated cytosol	9,710	95	5
Dialyzed cytosol	8,515	95	3
Cytosol preincubated at 32 C	10,360	94	6
Cytosol preincubated at 60 C	2,925	58	42
Dialyzed cytosol preincubated at 60 C	2,890	53	47

[a] 500-μliter samples of nuclei were incubated with additions as indicated in the presence of [^{3}H]TTP (100 μCi/ml) for 20 min. The Hirt supernatant fraction was analyzed as detailed in legend to Fig. 4.

since no specific changes at the product level were found.

As for viral DNA synthesis, it was of interest to find that cytosol from uninfected mouse cells (permissive for polyoma) as well as of uninfected monkey and hamster cells (both nonpermissive for polyoma) could substitute for the cytosol from productively infected mouse cells. This confirms conclusions drawn previously (5, 7) that no viral function is required for the continuation and completion of one round of viral DNA synthesis after it is initiated. They appear to be cellular functions, which are diffusible and, at least in part, found in the cytoplasm. The cytoplasmic factors do not seem to be species specific, at least for the three species tested, and are therefore not part of the system that determines whether a cell is permissive for virus reproduction or not. Largely due to the difficulty in working with chromosomal DNA, the analysis of the DNA synthesized in vitro in purified nuclei has not been sufficiently detailed to indicate what functions purified nuclei are lacking with regard to DNA synthesis. Making use of the relatively simple nature of polyoma DNA, we have been able to compare the viral DNA products made in purified nuclei in the presence and absence of cytosol, and we have noticed several striking differences. In purified nuclei there was a relative excess of label in short DNA chains (5S, Okazaki-type pieces) during pulse labeling, and even the long-term product (60 min) still contained 45% of the label in this size class. The amount of radioactivity accumulating in long DNA chains (≤16S, newly synthesized viral DNA strands) was greatly reduced in nuclei alone and restored in the reconstituted system to an extent corresponding to the concentration of cytosol added back. In terms of the proposed detailed mechanism of polyoma DNA synthesis in the unfractionated lysate (6), these observations can be interpreted in the following way. Two processes lead to the incorporation of labeled precursors into long chains: (i) the continuous extension of one progeny strand at the growing fork and (ii) the joining of short chains to the progeny strand on the other side. Both processes appear to be greatly reduced, whereas the synthesis of the short chains itself is the predominant synthetic event in purified nuclei. The accumulation of short chains under these conditions is not due to an irreversible damage to the nuclei or the viral DNA template, since they can be efficiently chased into long chains if cytosol is added back. The fact that the short chains pulse labeled in nuclei are smaller than in the complete lysate or the reconstituted system suggests that the defect in their processing affects the gap-filling step, thought to be necessary before they can be ligased to the growing strand (15, 17). Based on results to be published elsewhere, the possibility of discontinuous chain growth on both sides of the replication fork as a reason for the predominance of short chains in purified nuclei can be excluded; the self-complementarity of isolated short chains was not greater in nuclei (26%) than in the lysate (29%) or the reconstituted system (25%).

The synthetic pattern seen in purified nuclei resembles in many details the pattern found in the complete lysate, if partially inhibiting concentrations of 1-β-D-arabinofuranosyl cytosine 5-triphosphate (ara-CTP) are added to the in vitro reaction (Hunter and Francke, submitted for publication). The presence of ara-CTP also leads to an accumulation of short chains that are shorter than under noninhibited conditions. We have interpreted these results to the effect that the synthesis of short chains is accomplished by a relatively ara-CTP-resistant polymerase, whereas their processing is carried out by a polymerase relatively sensitive to the inhibitor, which is possibly the same one responsible for the continuous extension of the progeny strand on the other side of the fork. Based on the known ara-CTP sensitivity of isolated mammalian DNA polymerase (9; R. Fox and M. Goulian, personal communication), we had tentatively assigned the nuclear DNA polymerase (4) for the synthesis of short chains and the cytoplasmic enzyme for gap filling and continuous extension. The results reported here strongly support these assignments, since the

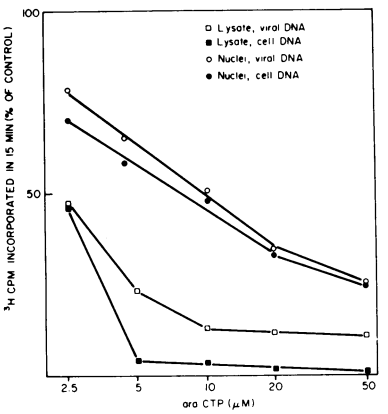

FIG. 8. *Ara-CTP sensitivity of DNA synthesis in the unfractionated lysate and in purified nuclei. 100-μliter incubations of lysate and of purified nuclei from ts1260-infected cells were prepared. dCTP was omitted from the lysate and reduced to 30 μM in the nuclei to compensate for the dCTP pool of the crude lysate (13). Incubations were carried out for 5, 15, 30, and 60 min in the presence of the indicated amounts of ara-CTP, and there was an additional time course without ara-CTP. The data presented were derived from the 15-min time points. The 5- and 30-min points (not shown) gave similar results.*

cytoplasmic polymerase is most likely the one that is removed by washing the nuclei. Based on these conclusions, one would predict that DNA synthesis in purified nuclei is less sensitive to inhibition by ara-CTP than in the complete system. This prediction was verified by the experiment shown in Fig. 8. The synthesis in nuclei can be inhibited completely by high enough concentrations of ara-CTP (data not shown) but is clearly more resistant at intermediate concentrations than the synthesis in the lysate. The reasons for the differential sensitivity to ara-CTP of cellular and viral DNA in the complete lysate are not fully understood.

We have tried to complement purified nuclei with isolated cytoplasmic polymerase from 3T3 cells (following the purification described by Sedwick et al. [19] up to and including the DEAE-cellulose step). Adding this enzyme over a wide range of concentrations to the nuclei with or without the heat-stable fraction from the cytosol had no effect on DNA synthesis. Thus, if the cytoplasmic DNA polymerase is one of the active components in the cytosol, which is strongly suggested by the data presented here, it is most likely not the only one. Bernard and Brent (2) have reported a stimulation DNA synthesis in HeLa cell nuclei by the addition of calf thymus DNA polymerase, but since the product was not characterized, the significance of the stimulation is not clear. A variety of other factors, possibly present in the cytosol, might be required in addition: DNA-binding proteins of the T4 gene 32 type (1); an unwinding protein (3); or DNA ligase, among others. A stimulation of DNA synthesis in HeLa cell nuclei by calf thymus histones has been reported by Hahn (10). We are presently pursuing the fractionation and identification of the cytoplasmic factors by using the system of nuclei from polyoma-infected mouse cells, since the complementation by cytosol is efficient and reproducible and the viral DNA can be characterized easily, to assess the specificity of active components obtained from the cytosol.

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