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In vitro synthesis of simian virus 40 DNA.

I. - Synthesis by a soluble extract from infected CV_1 cells.

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Summary. — Simian Virus 40 (SV40) DNA replication was studied *in vitro* using cell free extracts prepared from SV40 infected CV_1 cells. The cells were fractionated into a soluble cytoplasmic fraction and nuclei. The nuclei were lysed with high salt and used to prepare a soluble nuclear fraction. Both fractions displayed DNA polymerase activity as measured with activated calf thymus DNA. However, only the cytoplasmic fraction was active when SV40 DNA component I molecules were used as template. Under these conditions, the cytoplasmic extract was shown to catalyse the SV40 DNA dependent, *in vitro* incorporation of the four deoxyribonucleotides into DNA molecules which had, at both neutral and alkaline pH, the same sedimentation behavior as authentic SV40 DNA component I and component II molecules. Optimal Mg^{++} concentration was 5-8 mM. Incorporation of label into DNA component I molecules showed an initial lag of about 15 min., after which it was linear with time for up to 5 hrs at 32°. Incorporation into DNA component II molecules proceeded without obvious lag and reached a plateau after approximately 2 hrs of incubation. It is concluded that the cytoplasmic extract supports the *in vitro* synthesis of SV40 DNA and that DNA component II molecules appear to be a precursor to DNA component I molecules in the reaction. Labeling of viral DNA molecules was highly dependent on ATP and on an ATP generating system. In the absence of ATP and of the energy generating system, incorporation occurred but both template and newly synthesized DNA molecules were extensively degraded.

INTRODUCTION.

Several cell free systems have been described for the study of DNA replication in both uninfected and virus infected mammalian cells [1, 5, 6, 8, 10, 13, 16, 17, 19, 21, 24, 34]. These systems involve the use of either crude cell lysates or isolated nuclei.

Although much has been learnt from them regarding the elongation and termination of DNA synthesis, they have not allowed so far the characterization and purification of DNA replication factors in animal cells. One reason is that they are not able to support the *de novo* initiation of DNA synthesis (although initiation of Okasaki type fragments occurs) but can only elongate nascent DNA strands already initiated in the cell, prior to the preparation of the lysate. Another reason why these crude systems are not quite suitable is that replication of viral DNA's such as that of SV40 or polyoma virus is obscured by the continued occurrence of host cell DNA synthesis and/or repair. Last, but not least, these crude systems do not provide the investigator with the possibility of controlling the nature, the state, or the physical environment of the DNA template.

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It is however obvious that SV40 and polyoma virus could be ideal probes for the *in vitro* study of DNA replication in mammalian cells, since their mechanism of replication is well known from *in vivo* studies, and since elongation and termination of their replication appear to be entirely carried out by cellular gene products. The existence of the ts A mutants of both these viruses [7, 9, 29, 30], in which initiation of DNA synthesis is blocked *in vivo*, provides a unique means of studying the initiation of DNA replication in an animal cell system. Also, the study of SV40 or polyoma DNA replication *in vitro* might shed some light on the basic problem of why a cell is or not permissive to the replication of a given viral genome.

These considerations prompted us to prepare a soluble, cell free, DNA free and DNA dependent, extract from SV40 infected cells, rather than a cell free lysate. We shall describe a system which is able, when supplemented with SV40 DNA, to incorporate the four deoxyribonucleotides linearly with

time, during at least 5 hrs, into a material exhibiting the sedimentation properties of authentic SV40 DNA.

MATERIAL AND METHODS.

Cells and Viruses.

Subcloned CV_1 cell [22] were seeded in glass Roux bottles at a density of 5×10^6 cells per bottle and incubated at 37°C under growth medium (MEM supplemented with 10 per cent tryptose phosphate, 500 U/ml of penicillin G, 100 µg/ml of streptomycin sulfate, and 3.18 g glucose per liter added with 10 per cent calf serum). The medium was changed on day 3. On day 4, the cells were infected with wt SV40 [29], at a multiplicity of approximately 5 pfu/cell. Adsorption was for 2 hrs at room temperature, after which the cell culture was incubated for 65-70 hrs at 33°C under growth medium added with 2 per cent calf serum. In most experiments, the cultures were then transferred for 2 hrs to 41°C prior to collection of the cells. This procedure regularly yielded about 5×10^7 infected cells per Roux bottle.

The wt strain of SV40 was a gift from P. Tegtmeyer. Stocks of virus were prepared using plaque purified virus and infecting the cells at an input multiplicity of approximately 10^{-4} pfu/cell. Stocks were collected after 19-21 days at 33°C.

Preparation of DNA.

SV40 DNA component I was prepared according to Hirt [11] from CV_1 cells grown in 10 cm Petri dishes and infected 13 days previously with wt SV40 at an input multiplicity of approximately 10^{-4} pfu/cell. The low M.W. DNA obtained from the Hirt extraction supernatant was deproteinized 3 times with phenol and chloroform (1:1), precipitated with 2 vol. of 95 per cent ethanol at —20°C, then resuspended into 10^{-2} M Tris-HCl pH 7.4, 0.1 × SSC. The DNA was added with 100 µg/ml ethidium bromide and adjusted to a final density of 1.58 g/cm³ with solid CsCl. The mixture was centrifuged for 40 hrs at 40,000 rpm, 15°C, in the 50 titatium rotor of the Spinco. After centrifugation, the tubes were pierced and fractions collected dropwise. Those containing the band of superhelicoidal DNA (DNA component I) were centrifuged again to equilibrium through a new CsCl gradient in the presence of ethidium bromide. The resulting band of twice purified DNA component I was extracted 3 times with 1 volume isopropanol, dialyzed for approximately 16 hrs against a large volume of 10^{-2} M Tris-HCl pH 7.4, 10^{-4} M EDTA (TE buffer) and stored frozen at a concentration of 500 µg/ml.

Labeled SV40 DNA molecules were prepared similarly, except that the cell cultures were labeled with the required radioactive precursor ([³H] thymidine or ³²P, C.E.A., France) for 2 days before the extraction of the DNA. Labeling with 30 µCi/ml ³²P for that length of time typically yielded viral DNA with a specific activity of approximately 3×10^4 cpm/µg.

Activated calf thymus DNA (Boehringer) was prepared according to Wickner *et al* [33] and stored frozen at a concentration of 2 mg/ml.

Preparation of extracts.

Crude cytoplasmic extracts were prepared after Kidwell and Mueller [17], from 20 Roux bottles. The cells were scraped in warm phosphate buffered saline (PBS) with the aid of a silicone rubber, centrifuged at room temperature, resuspended in warm PBS, and sedimented again. The pellet was resuspended into 4 volumes of ice cold hypotonic buffer (buffer A : 10 mM potassium phosphate pH 7.8, 3 mM $MgCl_2$, 1mM EDTA, 2 mM dithioerythritol). The cells were allowed to swell for 10 min. at + 4°C then lysed by seven strokes of a tight fitting Dounce homogenizer (Kontes Glass Co). Immediately after lysis, isotonicity was restored to the cell extract through the addition of one-third volume of buffer B (240 mM Tris-HCl pH 8.0, 100 mM glucose, 400 mM NaCl, 1 mM EDTA, 2 mM dithioerythritol). Nuclei were then sedimented by centrifugation for 10 min. at 650 *g*, + 2°C. The resulting supernatant (cytoplasmic extract) was kept in ice while the pellet (nuclei) was resuspended into one half to one third the original volume of buffer A, dounced again, added with 1/3 volume of buffer B, and sedimented for 10 min. at 2 000 g through a 20 per cent w/w sucrose cushion in buffer C (see below). This second centrifugation step resulted in the fractionation of the crude nuclear homogenate into three layers : a soluble cytoplasmic fraction at the top, a particulate fraction (cell debris and unbroken cells) at the surface of the sucrose cushion, and a pellet (nuclei) at the bottom of the tube. The upper fraction was carefully removed and added to the preceding cytoplasmic extract. The cell debris were resuspended into a small volume of buffer A, homogenized again with the aid of the Dounce homogenizer, and sedimented at 2 000 *g* through a new 20 per cent sucrose cushion. The cytoplasmic fraction from

this last centrifugation was added to the preceding ones, and the nuclei were pooled with those from the preceding centrifugation.

The pooled soluble fractions representing the cytoplasmic extract were sedimented for 90 min. at 100,000 *g*, 2°C, in the 50 titanium rotor of the Spinco. The resulting supernatant (S100) was concentrated through precipitation by 80 per cent saturated ammonium sulfate in the cold. The extract was stored under the form of an ammonium sulfate pellet at —80°C.

The pooled nuclei were resuspended in a small volume of buffer C (60 mM Tris-HCl pH 8.0, 5 mM potassium phosphate pH 7.8, 3 mM $MgCl_2$, 2 mM dithioerythritol, 1 mM EDTA, 25 mM glucose, 100 mM NaCl) and treated with 0.25 per cent Triton X 100. They were then washed with buffer C after which they were extracted with 0.2 M potassium phosphate, pH 7.8 [28] in the presence of 2 M KCl, at a concentration of about 2×10^8 nuclei per ml. The resulting viscous lysate was clarified by centrifugation for 90 min at 100,000 *g*, 2°C, in the SW 50 rotor of the Spinco. The supernatant, representing the nuclear extract (N 100) was diluted 3 fold with buffer C, then precipitated with ammonium sulfate and stored as described for the cytoplasmic extract.

Standard conditions for in vitro *DNA synthesis.*

The nuclear (N 100) or cytoplasmic (S 100) ammonium sulfate precipitates were dialyzed against 25 mM Tris-HCl pH 7.5, 60 mM KCl, 5 mM β-mercaptoethanol, 2 mM EDTA, and 10 per cent glycerol (sample buffer) and in most experiments adjusted to 5 mg/ml protein with sample buffer. The dialyzed extracts could be stored at —80° for a couple of weeks without apparent loss of activity.

Standard assays for *in vitro* SV40 DNA synthesis were performed at 32°C in a final volume of 0.1 ml containing 40-50 μg/ml SV40 DNA component I. Concentration of the assay mixture was 20 mM Tris-HCl pH 7.7 (or 40 mM Hepes pH 7.8) 6 mM $MgCl_2$, 5 mM dithioerythritol, 100 μM each of dATP and dCTP, 50 μM each of dGTP and dTTP, 1 mM ATP, 6 mM phosphoenolpyruvate (PEP), 5 μg/ml pyruvate kinase, 250 μg/ml *E. coli* tRNA (Boehringer), 1.5 mM putrescine and 30 mM KCl. In addition, the extracts (.025 ml) contributed 6.25 mM Tris pH 7.5, 15 mM KCl, 1.25 mM β mercaptoethanol, 0.5 mM EDTA, and 2.5 per cent glycerol. SV40 DNA component I stored in TE buffer at — 80°C was diluted to 200 μg/ml with 10^{-2} M Tris-HCl pH 7.4, 5 mM $MgCl_2$, prior to use. It therefore contributed approximately 0.5 mM Mg^{++} and 1 mM Tris to the final concentrations. Labeling of the product of the reaction was through the use of [^{3}H] dTTP and/or [^{3}H] dGTP (New England Nuclear) at the indicated specific activities.

Bulk DNA polymerase activity was assayed in the presence of 20 μg activated calf thymus DNA at 37°C in a final volume of 0.1 ml. Assay mixture was 10 mM $MgCl_2$, 5 mM dithioerythritol, 20 mM Tris-HCl pH 7.7 or 8.8, with 100 μM each of dATP, dCTP and dGTP, 50 μM of [^{3}H]dTTP at the indicated specific activity, and 20 μg bovine serum albumine (BSA). One unit of DNA polymerase activity was defined as the amount of protein that incorporates 1 nmole of dTMP into DNA in 60 min. at the indicated temperature.

Incorporation of radioactive precursors was stopped by the addition of 0.2 ml ice cold 0.2 M Na pyrophosphate containing 10.0 μg/ml bovine serum albumin (BSA), followed by 5 ml of ice cold 5 per cent trichloracetic acid (TCA). The resulting precipitates were collected on GF/C glass fiber filters (Whatman), rinsed with approximately 20 ml 1 per cent TCA followed by 5 ml absolute ethanol, then dried under infrared light and counted in a toluene based scintillation fluid.

Analysis of the in vitro *labeled DNA.*

For the analysis of *in vitro* labeled products, incorporations were stopped by placing the tubes in ice and making their content 50 mM with respect to EDTA. In most experiments, SDS was then added to a final concentration of 0.3 per cent and the material was analyzed by alkaline sucrose gradient centrifugation through 11 ml 5-20 per cent gradients in 0.3 M NaOH, 0.7 M NaCl, 0.002 M EDTA. Centrifugation was for 12 hrs at 28.000 rpm 4°C, in the SW41 rotor of the Spinco. At the end of the centrifugation time, fractions were collected dropwise from the bottom of the tubes with the aid of a peristaltic pump and their content was processed for determination of TCA precipitable radioactivity.

In other experiments, the incubation mixtures were extracted first with phenol, then with phenol and chloroform (1:1) and the DNA was precipitated with ethanol. Part of the DNA was analyzed by neutral sucrose gradient centrifugation through 11 ml 5-20 per cent sucrose gradients in 0.01 M Tris-HCl pH 7.4, 1 M NaCl, 0.002 M EDTA. Centrifugation was for 12 hrs at 38,000 rpm, 4°C, in the SW41 rotor of the Spinco. Other portions of the deproteinized DNA sample were analyzed by sucrose gradient centrifugation at alkaline pH

as described above, or by equilibrium centrifugation in CsCl in the presence of 100 µg/ml ethidium bromide as described for the preparation of DNA's.

RESULTS.

SV40 infected cells were fractionated into a nuclear and cytoplasmic fraction as described under Material and Methods, and the 100,000 *g* supernatants from each (N100 and S100 respectively) were tested for their *in vitro* DNA polymerase activity after concentration by ammonium sulfate precipitation. Two tests were carried out, with either activated calf thymus DNA or SV40 DNA component I as template.

As will be reported in detail elsewhere (Cajean, Marty, Suarez and Girard, in preparation) both the S100 and N100 fraction displayed considerable DNA polymerase activity when assayed with calf thymus DNA, in agreement with the reported distribution of DNA polymerases in cell extracts [3, 4, 27, 28, 31, 32, 35]. However, only the S100 fraction displayed noticeable activity when assayed with SV40 DNA component I molecules.

TABLE I.

Requirements for SV40 DNA synthesis in vitro.

	S100 (µg proteins)	N100 (µg proteins)	Incubation medium	Incorporation (pmoles dTMP)
Experiment 1	43	32	complete	1.85
			omit SV40 DNA	.04
	72	—	complete	2.98
			omit SV40 DNA	.04
	48	—	complete	1.18
Experiment 2	48	42	complete	1.28
			omit dNTP	.52
			omit SV40 DNA	.04
			omit PEP and PK	1.72
			omit ATP, PEP and PK	2.38

Cytoplasmic (S100) and nuclear (N100) fractions were prepared from SV40 infected cells as described under Material and Methods and incubated for 30 min at 32°C. Complete system was 37 mM Hepes pH 7.8, 1 mM $MnCl_2$, 6 mM $MgCl_2$, 2 mM dithioerythritol, 1 mM ATP, 100 µM each of dATP, dGTP, dCTP, GTP, CTP and UTP, 200 µg/ml of BSA, 40 µg/ml of SV40 DNA component I, 50 µM[^{3}H]dTTP (250 cpm/pmole), and the indicated amount of extract in a final volume of 0.1 ml. The complete system in experiment 2 contained in addition 10 mM phosphoenolpyruvate (PEP) together with 5 µg/ml pyruvate kinase (P. K.).

This is illustrated in table I, which shows the result of an assay performed under the conditions described by Hunter and Francke [13]. Most of the activity measured in the presence of SV40 DNA was in the S100 extract.

This lack of activity in the nuclear extracts was not due to the conditions of the assay, since similar results were obtained under the assay conditions of Winnacker *et al* [34] of De Pamphilis and Berg [5] or under those described as standard conditions under the section Material and Methods. Neither was it due to the manner in which the cells were fractionated, since similar results were obtained when nuclei were prepared according to Spadari and Weissbach [28], or when they were prepared without detergent (results not shown).

Since only the cytoplasmic S100 fraction displayed substantial activity with SV40 DNA as template, the properties of the S100 system were investigated further. Incorporation with that system was fully dependent on the addition of SV40 DNA (table I : also, see figure 6 and 7), but only in part on the four deoxyribonucleoside-triphosphates (table I). The fact that the dNTP requirement is only partial may be due to the presence of residual amounts of dNTP's in the extract.

Incorporation was 99 per cent reduced when pancreatic DNase (10 µg/ml) was present during the assay. Pancreatic RNase (also 10 µg/ml) had no effect. The ^{3}H label was incorporated into a material which was resistant to alkaline hydrolysis, and resistant to 100 µg/ml RNase (30 min. at room temperature). The material was, on another hand, completely digested by DNase (also at 100 µg/ml) in 30 min. at room temperature (not shown). The amount of dNMP incorporated was proportional to the concentration of S100 extract used, at least in a range of approximately 0.1 to 1 mg/ml of protein. Higher concentrations of extract were found to be inhibitory.

As shown in figure 1, the system displayed a broad Mg^{++} concentration optimum, from approximately 3 to 8 mM. This was true not only of the gross incorporation of deoxyribonucleotides, as illustrated by figure 1, but also of the labeling of DNA molecules with the size and characteristics of SV40 DNA component I and component II. The addition of Mn^{++} to Mg^{++}, or the substitution of Mg^{++} by Mn^{++}, resulted in a somewhat variable stimulation of dTMP incorporation. It was found however that the presence of Mn^{++} during incubation could lead to extensive degradation of the SV40 DNA template, with the ensuing result that most of the label incorporated *in vitro* was in short

DNA fragments (not shown). We therefore refrained from using Mn^{++} in all the latter experiments.

Tris buffer (20 mM) could be successfully substituted for Hepes buffer, but potassium phosphate buffer, also at 20 mM, was inhibitory. Optimum pH was found to be from approximately 7.5 to 7.8 (not shown).

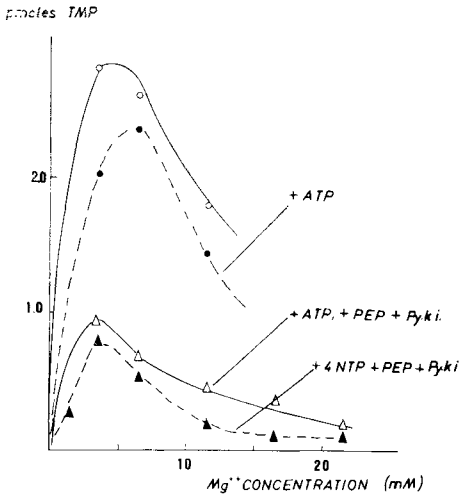

FIG. 1. — *Influence of the concentration of Mg^{++} on the synthesis of SV40 DNA* in vitro. A S100 fraction, prepared from SV40 infected cells, was incubated at 32° for 35 min. as described in the legend to table I (experiment 2), with variable concentrations of Mg^{++} a) in complete medium (▲- - -▲) ; b) omitting GTP, CTP, and UTP (△—△) ; c) omitting the same and the energy generating system (●- - -●) ; or d) as in c) but without ATP (○—○). The S100 extract contributed 0.72 mg of protein per ml in the assay.

The influence of the four ribonucleoside triphosphates was next investigated. That of GTP, CTP and UTP was found to be negligible, because their addition was without major effect on either the gross incorporation of dNTP's by the system (fig. 1) or the nature of the DNA product (see for example figure 7 in the accompanying paper). The importance of ATP and of an ATP generating system, on the other hand, was capital : surprisingly, the addition of ATP and of an energy generating system decreased the incorporation of dTMP 2 to 3 fold (table I and fig. 1).

The effect of ATP on the nature of the DNA labeled during the incubation *in vitro*, and on the fate of the SV40 DNA component I used as template in the assay, was therefore investigated. For that purpose, SV40 DNA component I which had been labeled *in vivo* with ^{32}P, was used as template in an assay using [^{3}H]dTTP to label the product, either in the presence or absence of 1 mM ATP. At the end of the incubation period, the ^{32}P labeled DNA molecules and the ^{3}H labeled product were analyzed by sucrose gradient centrifugation at either alkaline or neutral pH (fig. 2). In the absence of ATP (right hand side panels in figure 2) the ^{32}P labeled template DNA molecules were considerably degraded as judged from the fact that they sedimented as 8-12S material at neutral pH (closed circles, panel B), and as 4-8S fragments at alkaline pH (closed circles, panel D). Similarly, the overwhelming majority of the product labeled

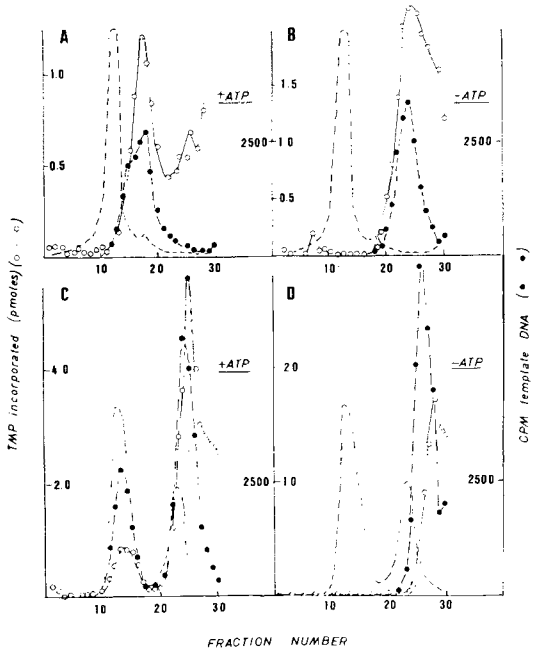

FIG. 2. — *Influence of ATP on the final size of the SV40 DNA template and product molecules.* A S100 extract was incubated as described in the legend to table I, except that GTP, CTP and UTP were omitted, and that the SV40 DNA component I used as a template was labeled with ^{32}P. One assay was done in the presence of 1 mM ATP (left panels), the other in the absence of ATP (right panels). After 60 min. incubation at 32° the reaction was stopped by the addition of EDTA and part from each incubation mixture was analyzed by sucrose gradient centrifugation at either neutral pH (panels A and B) or alkaline pH (panels C and D). Symbols : ○—○ : pmoles acid precipitable [^{3}H]-dTMP ; ●- - -● : cpm ^{32}P DNA. The dashed line refers to the sedimentation of a sample of the ^{32}P template DNA which had not been incubated and was centrifuged in parallel.

in vitro with [^{3}H]-dTMP sedimented as fragmented material at less than 12S at neutral pH (panel B in fig. 2) and at approximately 4S at alkaline pH (panel D).

In the presence of ATP, on the opposite, most of the ^{32}P labeled DNA sedimented as unit length

DNA molecules (left hand side panels in figure 2). ATP was therefore apparently required to maintain the integrity of the DNA template molecules.

ATP also conditioned the form of the DNA product obtained. Approximately 50 per cent of the [^{3}H]-dTMP labeled product made *in vitro* by the S100 system in the presence of ATP sedimented at about 18S at neutral pH (open circles, panel A in figure 2). Most of this label was in DNA component II molecules, as judged from its behavior at alkaline pH, but a definite amount was recovered in DNA component I molecules (open circles, panel C).

However, these molecules lacked superhelicity, since they sedimented at slower than 21S at neutral pH (panel A in figure 2) and since their density in the presence of ethidium bromide was heavier than that of a DNA component I marker (see fig. 3).

Similarly, after incubation with the S100 extract in the presence of ATP, the sedimentation pattern of the ^{32}P labeled template DNA was shifted at neutral pH from a homogeneous 21S peak (dashed line in panel A) to a heterogeneous peak at approximately 18S (closed circles in panel A). At alkaline pH, only 1/3 of the molecules still sedimented at 53S as a DNA component I marker (closed circles in panel C), while the remainder sedimented at 16-18S as DNA component II molecules. These results show that in the presence of ATP, 1/3 of the template DNA molecules were converted into relaxed circular DNA molecules [2, 15, 36] and 2/3 into nicked (component II) DNA molecules.

These results were ascertained by determining the density distribution in CsCl in the presence of ethidium bromide of the DNA labeled *in vitro* using [^{3}H]-dTTP. The DNA products labeled in the presence of ATP exhibited a bimodal distribution, with 78 per cent of the product having a density similar to that of SV40 DNA component II molecules, and 22 per cent a density heavier than that of authentic component I molecules (fig. 3), corresponding to relaxed DNA component I molecules [36]. When ATP was omitted from the incubation mixture, the DNA showed a unimodal distribution, with the totality of the label in the band corresponding to linear DNA molecules (not shown).

Therefore, although the addition of ATP slowed the incorporation of dNMP's (fig. 1), its presence was essential to preserve the integrity of both template and newly synthesized molecules. No intact component I molecules could be obtained in the absence of ATP, whereas in the presence of ATP as much as 25 per cent of the nucleotides incorporated, sedimented at 53S at pH 13 and banded with a density characteristics of covalently closed relaxed circular DNA molecules in the presence of ethidium bromide.

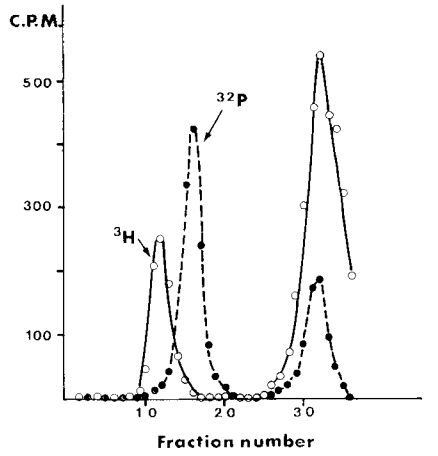

FIG. 3. — *Analysis of the* in vitro *labeled DNA by equilibrium centrifugation in CsCl in the presence of ethidium bromide.* Part of a reaction mixture incubated for 60 min. at 32° with [^{3}H] dTTP (500 cpm/pmole) was analyzed together with a ^{32}P labeled SV40 DNA marker by equilibrium centrifugation in the presence of ethidium bromide as described in Material and Methods. After centrifugation, fractions were collected and assayed for TCA precipitable radioactivity. Symbols : ●---● : ^{32}P SV40 DNA marker ; ○—○ : [^{3}H] dTMP labeled material. The density of the marker DNA component I was 1.580 g/cc.

Degradation of the template and newly formed molecules, in the absence of ATP, was tentatively attributed to endonuclease activity. Therefore an attempt was made at inhibiting endonucleases by addition of KCl or *E. coli* tRNA to the S100 system.

Figure 4 shows that in the presence of *E. coli* tRNA, overall incorporation of dTMP by a S100 extract was decreased, but the final product was much more homogeneous and of larger size than that made in the absence of tRNA. The effect of KCl alone or combined with tRNA on the overall incorporation of dTMP are illustrated in figure 5. Both compounds lowered DNA polymerase activity, but to a different extent depending upon the nature of the DNA used as template in the assay. Bulk DNA polymerase activity, as measured with activated calf thymus DNA, was not inhibited by the addition of tRNA, and was progressively but slowly inhibited by KCl concentrations above 20 mM (triangles in figure 5). But dTMP incorporation using SV40 DNA as template was decreased 60 per cent by the mere addition of tRNA and was extre-

mely sensitive to addition of salt (circles in figure 5). This shows that the decrease in incorporation into SV40 DNA induced *in vitro* by tRNA and /or KCl, is not so much due to the inhibition of the DNA polymerase(s) in the S100 extract as to the inhibition of contaminating endonucleases.

The effect of varying concentrations of KCl on the size of the *in vitro* labeled product and on the fate of the SV40 component I template were also studied. It was found that the combination of 30-50 mM KCl together with 250 μg/ml of tRNA was optimal, as judged from the amount of labeled dTMP incorporated per mg protein of S100, and from the percentage of that label which was recovered as unit length DNA molecules at pH 13 (not shown). All subsequent experiments were therefore performed using these concentrations of KCl and tRNA. In addition, we found, at times, a slight enhancement of the labeling of high M.W. DNA when using 1.5 mM putrescine in the assay. Putrescine was therefore systematically added in all subsequent assays.

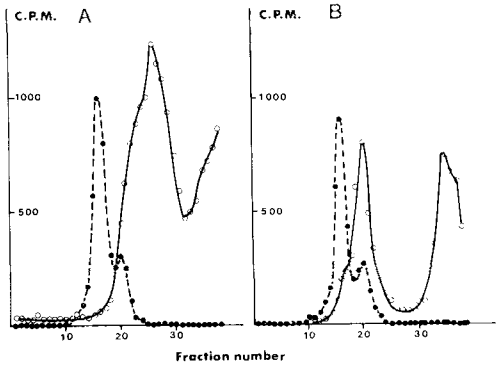

FIG. 4. — *Influence of tRNA on the size of the DNA made* in vitro. The products of a 60 min. incubation at 32° in the presence (panel B) or absence (panel A) of 250 μg/ml of *E. coli* tRNA were analyzed together with a ^{32}P labeled SV40 DNA marker by centrifugation at neutral pH. The assay was as described under Material and Methods using 20 mM Tris pH 7.7, and no KCl. Symbols : ●- - -● : ^{32}P SV40 DNA marker ; ○—○ : [^{3}H] dTMP labeled material.

Using these standardized conditions, the influence of the concentration of template DNA was next studied. An assay was carried out using 1.05 mg/ml of S100 proteins and 10, 20, 40 and 80 μg/ml of SV40 DNA component I molecules. The DNA product labeled after 150 min. incubation *in vitro* was analyzed part at pH 13 (fig. 6), and part at neutral pH (fig. 7). As seen in figure 6 (panel E), both the amount of label recovered in covalently closed circular DNA molecules sedimenting at 53S at pH 13 (fractions 9 to 15 in panels A-D), and that in unit length DNA molecules sedimenting at about 18S at the same pH (fractions 22 to 28) varied linearly with the concentration of template DNA, at least in the range from 20 to 80 μg/ml of DNA. The proportion of label recovered as covalently closed circular DNA molecules (53S) also increased with increasing template concentration. The amount of label in fragmented DNA material (about 4S at pH 13) was however independent of the concentration of template DNA. Thus, the more concentrated the DNA template in the assay, the greater the proportion of label in closed circular DNA molecules.

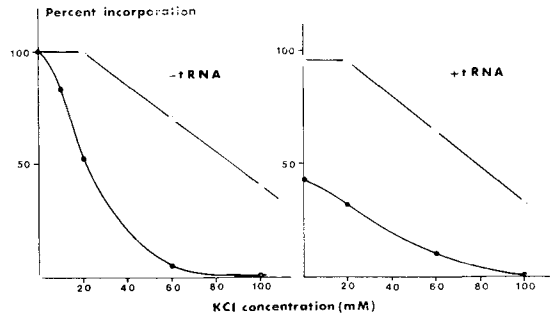

FIG. 5. — *Influence of KCl on the DNA polymerase activity of a S100 extract.* The effect of KCl was studied in both a standard 30 min. assay for DNA polymerase activity, using activated calf thymus DNA as the template (△), and in a 35 min. assay for SV40 DNA synthesis, using viral DNA component I as the template (●). Conditions for the latter were as in the legend to figure 4. Both assays used 48 μg of S100 extract proteins. Increasing concentrations of KCl were tested in the absence (left panel) or in the presence (right panel) of 250 μg/ml of *E. coli* tRNA. Control incorporation in the absence of KCl and tRNA were taken as 100 per cent incorporation : actual figures were 38 pmoles with calf thymus DNA and 10 pmoles with SV40 DNA.

The DNA product was also analyzed at neutral pH (fig. 7). In agreement with the above results, the amount of label recovered in DNA sedimenting faster than 16S (fractions 10-20 in panels A-D of figure 7) varied linearly with the concentration of template DNA (panel E). Part of the material labeled *in vitro* now sedimented at 21S, in the same position as authentic viral DNA component I. This is quite different from what was observed in the experiment of figure 2 where the DNA which was 53S at pH 13 was about 18S at neutral pH. *In vitro* labeled 21S DNA was reproducibly obtained when using the assay conditions described above (see figure 8, and accompanying paper). This is probably the consequence of the improved assay conditions, in particular of the use of tRNA, KCl and putrescine.

The time course of SV40 DNA synthesis *in vitro* was then determined, using a template concentration of 50 μg/ml of SV40 DNA component I in the presence of 46 mM KCl, 250 μg/ml of tRNA and 1.5 mM putrescine (figure 8). At the end of each incubation period, the reaction was stopped by the addition of EDTA and SDS. The DNA was extracted with phenol and precipitated with ethanol. Part of the precipitate was analyzed by sucrose gradient centrifugation at neutral pH, whereas another part was analyzed at alkaline pH. The sedimentation pattern of the product labeled after 300 min. of incubation *in vitro* is shown as an example in the left hand panels of figure 8. At neutral pH (panel A) three peaks of labeled material were identified at 21S, 18S and 4S respectively. At alkaline pH (panel C) three peaks of material were identified at 53S, 16-18S and 4S. These sedimentation coefficients were determined from the sedimentation profile of a ^{32}P labeled viral DNA marker centrifuged in parallel.

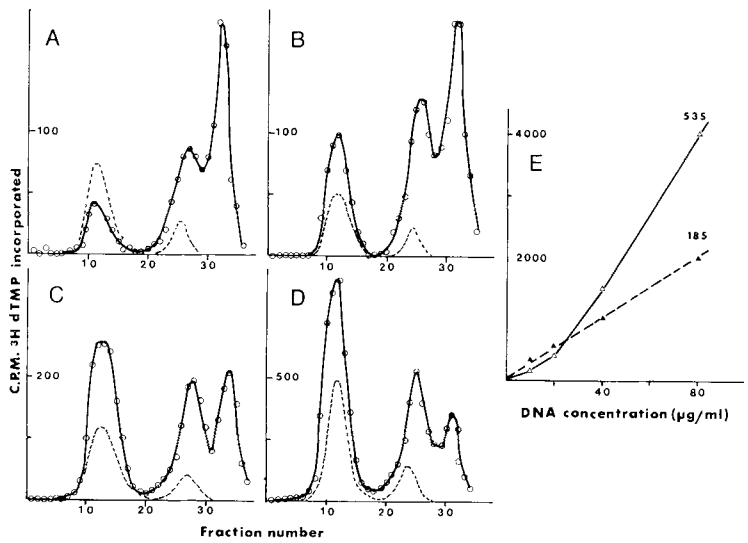

FIG. 6. — *Influence of the concentration of SV40 DNA template on the nature of the* in vitro *product analyzed at alkaline pH.* A standard incorporation assay was set as described in Material and Methods using 104 μg of a S100 extract protein, and variable amounts of template SV40 DNA component I. Both dTTP and dGTP were labeled with tritium. The reaction was stopped after 150 min. incubation at 32° by the addition of EDTA and SDS, followed by phenol extraction. The ^{3}H-labeled DNA's were precipitated with ethanol, resuspended into 0.1 × SSC, added with [^{32}P] SV40 DNA_1 as a marker, and part of each sample was analyzed by sucrose gradient centrifugation at alkaline pH : cpm ^{3}H (○—○) and cpm ^{32}P (- - -). The concentrations of SV40 DNA template in the different assays were : panel A : 10 μg/ml ; panel B : 20 μg/ml ; panel C : 40 μg/ml ; panel D : 80 μg/ml. The amount of ^{3}H radioactivity recovered in 53S molecules (△—△) and in 18S molecules (▲- - -▲) in the various gradients was plotted as a function of template concentration in panel E.

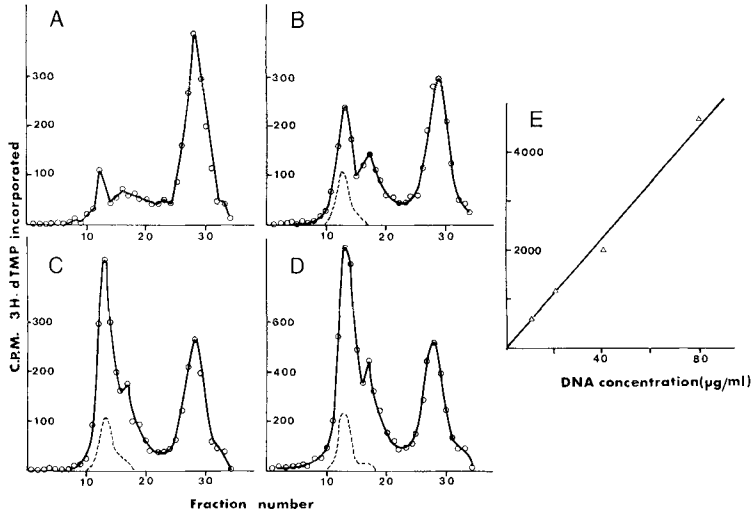

FIG. 7. — *Influence of the concentration of SV40 DNA template on the nature of the* in vitro *product analyzed at neutral pH.* Part of the SV40 DNA synthesized *in vitro* in the preceding experiment (fig. 6) was analyzed by sucrose gradient centrifugation at neutral pH. Symbols : ○—○ : [^{3}H] dTMP ; - - - - : [^{32}P] DNA marker. The amount of ^{3}H radioactivity recovered in material sedimenting faster than 15S was plotted as a function of template concentration in panel E (△—△).

The amount of radioactivity recovered in the different peaks of the gradients after various times of incubation were plotted against time to determine the time course of the *in vitro* labeling of the three DNA species (fig. 8 panels B (neutral pH) and D (alkaline pH)). After short times of incuba-

tion, the labeling of small M.W. DNA fragments (4S) was predominant, but plateaued rapidly. Accumulation of label into 18S molecules was more sustained, but also eventually plateaued after 120 min. By contrast, after an initial lag of 15-20 min., label accumulated linearly into closed circular DNA molecules (21S, neutral pH, panel B, and 53S, alkaline pH, panel D). No evidence for transient appearance of replicative intermediate (R.I.) molecules, which would have been 25S at neutral pH [14, 18, 26] could be found even after the shortest times of incubation examined.

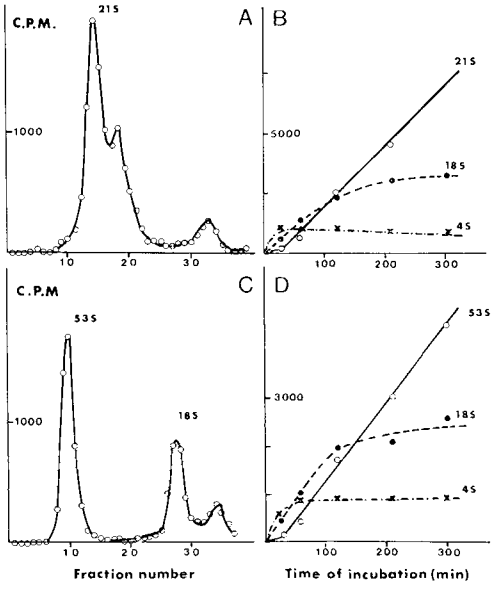

FIG. 8. — *Time course of SV40 DNA synthesis* in vitro. A standard assay system (see Material and Methods) was incubated for varied length of times, in the presence of both [^{3}H]-dTTP and [^{3}H]-dGTP, after which the DNA labeled *in vitro* was analyzed as described in the legend to the preceding figures. The behavior of the 300 min. sample is shown here as an illustration : part of that sample was analyzed at neutral pH (panel A), part at pH 13 (panel C). The peaks labeled 21S in panel A, and 53S and 18S in panel B, refer to the sedimentation position of a ^{32}P labeled SV40 DNA marker added to the sample prior to centrifugation. The number of counts recovered from each of these gradients as 21S, 18S and 4S material at neutral pH, and as 53S, 18S and 4S material at alkaline pH, are plotted as a function of time of incubation in panels B and D respectively. Note that different amounts of material were analyzed in the two sets of gradients.

It should be pointed out that, though much higher than in the nuclear extract, the activity of the cytoplasmic fraction (S100) of the infected cells was quite low under the conditions of the assay. For example, it was barely detectable at the step before ammonium sulfate precipitation (although gross DNA polymerase activity was easily detectable at that stage using activated calf thymus DNA as template). The efficiency of the system after concentration by ammonium sulfate was calculated from the data obtained in the time course experiment shown in figure 8. A total of 24 pmoles each of dTMP and dGMP were incorporated into SV40 DNA component I and component II molecules in the course of 300 min. Since the amount of template SV40 DNA_I in the assay was 5 µg, i.e. approximately 1.4 pmole DNA, it follows that on the average, about 75 deoxyribonucleotide residues were incorporated per molecule of template DNA. The ratio of newly synthetized DNA to template DNA was therefore only approximately of 0.6 per cent. On a mg of protein basis, the system exhibited a specific activity of 0.2 unit/mg with SV40 DNA as template (0.2 nmole deoxyribonucleotide incorporated in 60 min. per mg of protein) which is about 15 times less than that it exhibited with activated calf thymus DNA. The reason why the activity with SV40 DNA was so low likely relates to the lack of free natural 3'OH ends in the viral DNA molecule used as template, and to the slow rate at which free ends could be formed in the assay, for example as a consequence of endonucleolytic attack.

DISCUSSION.

Since the replication of SV40 DNA takes place in the nucleus of the infected cell *in vivo*, and since elongation and termination of SV40 DNA replication can be carried out by isolated nuclei *in vitro* [5, 6, 24], the obvious approach to the obtention of a soluble, cell free replication system for SV40, should have been to start from the nuclear fraction of SV40 infected cells.

However, the experiments reported here show that the nuclear extract from SV40 infected cells had very little detectable DNA polymerase activity when supplemented *in vitro* with SV40 DNA component I molecules as template. By contrast, the cytoplasmic extract from the same cells displayed a definite ability to incorporate deoxyribonucleotides under the same conditions.

The lack of activity of the nuclear fraction was not due to the loss or inactivation of nuclear DNA polymerases, since, when assayed with an activated DNA template, the nuclear extracts were at least 50 per cent as active as the cytoplasmic extracts (per mg of protein). For example, with activated calf thymus DNA as a template, the nuclear (N100) fraction from the experiment shown in table I displayed an activity of 1.82 units DNA polymerase per mg of protein, whereas the

cytoplasmic (S100) fraction displayed an activity of 2.50 units/mg. Still, with SV40 DNA component I as template, the same nuclear fraction was 6-10 times less active than the cytoplasmic fraction.

A possible explanation for this lack of activity is that critical enzymes or factors required for the synthesis of SV40 DNA *in vitro* leaked out from the nuclei during the preparation of the cytoplasmic extracts. Several nuclear enzymes are known to leak out from nuclei when aqueous procedures are used for the fractionation of the cell. For example, class C mammalian RNA polymerase is preferentially recovered in the cytoplasmic fraction of cell extracts [12]. Also, elongation of nascent DNA chains by isolated nuclei from HeLa cells requires the presence of the soluble protein fraction from the cytoplasm [10, 16, 17]. Similarly, most of the synthesis of viral DNA component I which can take place *in vitro* in a SV40 or polyoma virus infected cell lysate, is lost when the nuclei are separated from the cytoplasmic components, but restored when the nuclei are incubated in the presence of the soluble protein fraction from the cytoplasm [5, 8, 21, 24]. It is known also that SV40 early proteins, such as T antigen, which are located inside the cell nucleus *in vivo,* are easily recovered from the cytoplasmic fraction of cell extracts *in vitro* [23, 30]. The reason why the nuclear extracts from SV40 infected cells we used failed to replicate exogenously added SV40 DNA, could therefore be that they had lost, into the cytoplasm, one or several of the factors required to carry out the *in vitro* synthesis of covalently closed circular DNA molecules. Another possibility is that the environment in which the nuclear extracts were tested lacked some cofactor or activator which is required for the initiation of viral DNA replication, and the nature of which is presently unknown.

Since only the cytoplasmic fraction of the cell showed activity with SV40 DNA as a template, we concentrated on the study of that activity. Incorporation with that system was rather remarkable, both by the nature of the labeled product and by the prolonged duration of the incorporation. Indeed, the S100 extract was able to incorporate the monophosphate moiety of the four deoxyribonucleoside triphosphates into a covalently closed, circular DNA product which, except for the superhelicity of the molecule, was indistinguishable from authentic SV40 DNA_I. This reaction could be made to last at a constant rate for at least 300 min., close to 60 per cent of the DNA product being recovered as relaxed DNA component I molecules, and about 30 per cent as DNA component II (see fig. 8).

The only obvious requirement for the reaction to last that long was that for a constant supply of ATP. In the absence of ATP, all template molecules were degraded to small M.W. fragments, demonstrating extensive attacks by endonucleases, and the reaction stopped progressively. In the presence of ATP, on the other hand, the template molecules did not suffer more than a few single strand breaks, since not more than 60 per cent (fig. 2) and usually only 25-30 per cent (see accompanying paper) were recovered as DNA component II molecules. None appeared to be fragmented to small M.W. fragments. This suggests that in the presence of ATP, either the activity of the endonucleases of the extract was inhibited, or, rather, random single strand attacks of the circular template DNA molecules by the endonucleases of the extract still occurred, but were quickly repaired by the cell DNA ligase. It is known that mammalian cell DNA ligase is dependent on ATP [25], which would explain why high levels of ATP were constantly required to prevent excess degradation of the DNA. This, in turn, suggests that the activity of the S100 system is mostly that of a repair-like process involving the breakdown and resynthesis of template DNA molecules, but not net synthesis of DNA. That this is indeed the case will be demonstrated in the following paper.

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RÉSUMÉ.

Dans le but d'étudier la réplication du DNA dans un système acellulaire dérivé de cellules animales en cultures, nous avons choisi d'étudier la synthèse du DNA du SV40 en extraits de cellules CV_1 infectées par le virus. On a comparé l'activité d'extraits cytoplasmiques et d'extraits nucléaires. En présence de DNA de thymus de veau activé utilisé comme matrice, les deux extraits manifestent des activités DNA polymérase comparables. Par contre en présence de DNA de SV40 forme I comme matrice, seul l'extrait cytoplasmique manifeste une activité significative. Nous avons donc concentré notre attention sur le système cytoplasmique. Ce système catalyse l'incorporation des 4 désoxyribonucléotides dans un produit qui possède les mêmes propriétés de sédimentation, tant à pH neutre qu'à pH alcalin, que les formes I et II du DNA viral. Les formes I synthétisées *in vitro* sont toutefois dépourvues de superhélicité. Le système est exigeant en Mg^{++}, en DNA, en désoxyribonucléotides, en ATP et en système générateur d'énergie. La cinétique d'incorporation de dTMP dans le DNA forme I montre un temps de latence d'environ 15 mn, après quoi elle devient linéaire. Elle se maintient à taux constant jusqu'à plus de 5 heures à 32°C. La cinétique d'incorporation de dTMP dans le DNA forme II ne montre pas de latence. Elle atteint un plateau après quelque 2 heures à 32°C. Ceci suggère que

les molécules de forme II sont des précurseurs des molécules de forme I. L'ATP est indispensable pour maintenir l'intégrité des DNAs durant la réaction.

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