In vitro Synthesis of Herpes Simplex Virus DNA in Nuclei Isolated from Infected BSC 1 Cells

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SUMMARY

The synthesis of herpes simplex virus DNA in isolated nuclei under in vitro conditions was found to be dependent on the addition of ATP and an ATP generating system to the reaction mixture. In vitro DNA synthesis was stimulated and prolonged when p-hydroxymercuribenzoate was added to the isolated nuclei. Under these improved conditions virus DNA molecules which were initiated in vivo were completed in vitro, but most of the DNA molecules synthesized in vitro sedimented in sucrose gradients more slowly than herpes virion DNA. Denaturation of the in vitro labelled DNA molecules produced short single-stranded labelled DNA chains. Thus, under our improved in vitro conditions there was prolonged synthesis of DNA at a high rate, with the formation of both complete and incomplete virus DNA molecules.

INTRODUCTION

Studies on in vitro synthesis of herpes simplex virus (HSV) DNA in nuclei isolated from infected cells (Fine & Ludwig, 1972; Radsak, 1973; Bell, 1974; Biswal & Murray, 1974; Becker & Asher, 1975; Bolden, Aucker & Weissbach, 1975; Kolber, 1975; Shlomai & Becker, 1975) showed that 3H-thymidine triphosphate is incorporated into HSV DNA molecules by a process that is thought to be semiconservative DNA biosynthesis (Kolber, 1975). The conditions used in the different reports for the study of DNA synthesis in HSV-infected nuclei varied considerably. However, in most of the systems, DNA synthesis occurred for short periods only.

In our studies (Becker & Asher, 1975; Shlomai & Becker, 1975) we used a potassium phosphate buffer which selectively prevented the synthesis of cellular DNA but allowed virus DNA to be synthesized in the isolated nuclei for a period of 60 min. In contrast, the use of HEPES buffer (Kolber, 1975) allowed DNA synthesis to continue for a period of 20 min only and was followed by active degradation of the in vitro synthesized DNA (Kolber, 1975; Y. Becker & Y. Asher, unpublished results). In the present study improved conditions for in vitro DNA synthesis were investigated in order to prolong the synthesis of virus DNA and to obtain an undegraded virus DNA product in vitro. The synthesis of HSV DNA was found to be dependent on the presence of ATP and an ATP generating system, but not on the preparation of nuclei in the presence of calcium ions. Addition of p-hydroxymercuribenzoate, a sulphydryl reagent, to the reaction mixture in the presence of dithiothreitol, markedly stimulated the synthesis of virus DNA.
METHODS

Cells and virus. BSC 1 cell monolayers (4 x 10^6 cells/milk bottle) were infected with the HF strain of HSV type 1 at 10 p.f.u./cell and the cells were incubated at 37°C for 12 to 14 h.

Preparation of nuclei from HSV-infected cells. The infected cells were scraped into reticulocytite standard buffer (RSB: 0.01 M-tris-HCl, pH 7.7, 0.01 M-KCl, 0.015 M-MgCl₂, 10⁻³ M-dithiothreitol) homogenized in a glass Dounce homogenizer and centrifuged for 2 min at 800 rev/min in a PR-2 refrigerated centrifuge. The nuclear pellet was washed in a phosphate buffer (80 mM-potassium phosphate, pH 7.4, 0.25 M-sucrose, 10⁻³ M-dithiothreitol) and resuspended at a concentration of 4 x 10⁶ nuclei/100 μl in the same buffer. Unless stated otherwise, all the above buffers contained 0.4 mM-CaCl₂.

In vitro conditions for virus DNA synthesis in isolated nuclei. Each 600 μl of reaction mixture contained: 8 x 10⁶ nuclei in 80 mM-potassium phosphate buffer, pH 7.4, 250 mM-sucrose (RNase free), 6 mM-MgCl₂, 0.4 mM-CaCl₂, 1 mM-dithiothreitol, 0.04 mM each of dATP, dGTP, dCTP and 0.002 mM of ³H-TTP; 1 mM-ATP, 5 mM-phosphoenol pyruvate, 15 μg/ml pyruvate-kinase and 5 mM-ethyleneglycol-bis(β-aminoethyl ether) N,N’-tetra-acetic acid (EGTA). Where indicated the mixture contained one or more of the following: 0.04 mM each of ATP, CTP, GTP, UTP, 1 mM-p-hydroxymercuribenzoate (PHMB), 10 mM-(NH₄)₂SO₄, 0.0087 mM-³H-UTP.

CsCl density gradients. At different times after initiation of DNA synthesis in isolated nuclei, SSC buffer was added to a final concentration of 1 x SSC (0.15 M-NaCl, 0.015 M-sodium citrate). In addition, 0.5% sodium lauryl sulphate, and 0.3 mg/ml Pronase (free of nucleases, 90,000 units/ml) were added. The lysates were incubated at 37°C for 5 to 7 h. CsCl dissolved in TE buffer (0.01 M-tris-HCl, pH 7.5, 0.001 M-EDTA) was added to each DNA preparation to bring the density to 1.70 g/ml. The preparations were centrifuged in polyallomer tubes in the 50 Ti rotor of the Beckman preparative ultracentrifuge at 35,000 rev/min for 48 h at 20°C. The gradients were collected dropwise from the bottom of the tube, and the trichloroacetic acid (TCA) precipitable radioactivity in each fraction was determined. The buoyant densities were determined by measuring the refractive indices of various fractions in the Bausch and Lomb refractometer. Virus DNA was separated from cellular DNA according to their different buoyant densities (1.718 and 1.700 g/ml, respectively).

Sedimentation analysis in sucrose gradients. Virus DNA preparations, purified by centrifugation in CsCl density gradients, were diluted in TEN buffer (0.01 M-tris-HCl, pH 8.0, 0.001 M-EDTA, 1.0 M-NaCl) and carefully layered on to linear 5 to 20% (w/v) sucrose gradients prepared in TEN buffer. DNA extracted from herpes simplex virions or from T2-colicphage, was used as a marker. The gradients were centrifuged in polyallomer tubes at 12,500 rev/min for 12 h at 20°C, in the SW27 rotor of the Beckman L-2 ultracentrifuge. The gradients were collected dropwise and the TCA precipitable radioactivity in each fraction was determined. Sedimentation analyses in alkaline sucrose gradients were carried out as above using an alkaline DNA ‘buffer’ (0.2 N-NaOH, 0.8 M-MNaCl, 0.001 M-EDTA).

Materials. The growth medium used for cell cultures was Dulbecco’s modified Eagle’s medium containing 5% calf serum. Sucrose (RNase free) from Schwartz/Mann, Orangeburg, New York, U.S.A.; dithiothreitol, ribonucleoside triphosphates, deoxyribonucleoside triphosphates, phosphoenolpyruvate (PEP), pyruvate kinase (PK), ethyleneglycol-bis (β-amino ether) N,N’-tetra-acetic acid (EGTA) and p-hydroxymercuribenzoate (PHMB) from Sigma, St Louis, MO, U.S.A.; methyl ³H-thymidine triphosphate ammonium salt (sp. act. 47 Ci/mmol) from the Radiochemical Centre, Amersham, England; uridine 5’-triphosphate, tetrasodium salt (5,6-³H), 37.5 Ci/mmol, from New England Nuclear, Boston.
HSV-DNA synthesis in isolated nuclei

Fig. 1. Factors affecting in vitro DNA synthesis. HSV-infected BSC 1 monolayers were scraped into RSB at 14 h p.i. and homogenized in a glass Dounce homogenizer. The nuclei were prepared either in the presence (curves 2, 4, 6) or absence (curves 1, 3, 5) of Ca\(^{2+}\), as described in Methods. Each 600 \(\mu\)l of reaction mixture contained 8 \(\times\) 10\(^6\) nuclei in 80 mM-potassium phosphate buffer, pH 7.4, 250 mM-sucrose, 6 mM-MgCl\(_2\), 1 mM-dithiothreitol, 0.04 mM each of dATP, dGTP, dCTP and 0.002 mM of \(^{3}\)H-TTP. The reaction mixtures were prepared with or without the addition of CaCl\(_2\), ATP, phosphoenol pyruvate (PEP) pyruvate kinase (PK) or the four ribonucleoside triphosphates (rN). Samples were withdrawn at the indicated time intervals and the TCA precipitable radioactivity in each sample was determined.

Mass., U.S.A. CsCl from E. Merck, Darmstadt, Germany, and Pronase from Calbiochem, U.S.A.

RESULTS

In vitro DNA synthesis in HSV-infected nuclei incubated in phosphate buffer

Incubation of nuclei from HSV-infected cells in the presence of phosphate buffer and the four deoxyribonucleoside triphosphates resulted in the synthesis of virus DNA as well as cellular DNA (Becker & Asher, 1975; Shlomai & Becker, 1975). Under these conditions, in vitro DNA synthesis continued for a period of 60 min and was followed by partial DNA degradation as shown in Fig. 1, curve 1, and as previously reported (Becker & Asher, 1975).

Effect of Ca\(^{2+}\)

Qasba (1974 a,b) suggested that calcium ions were required during the preparation of nuclei from SV40-infected cells to obtain effective in vitro DNA synthesis. Preparation of nuclei from HSV-infected BSC 1 cells in the presence of Ca\(^{2+}\) had no stimulatory effect on the in vitro synthesis of DNA (Fig. 1, curve 2). Calcium ions used in the preparation of nuclei from polyoma infected cells were removed from the in vitro reaction mixture by the
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Fig. 2. In vitro synthesis of RNA and DNA in the presence and absence of α-amanitin. Nuclei from HSV-infected BSC 1 cells were prepared at 14 h p.i. as described in Methods, and incubated at 37°C in the reaction mixture containing 250 mM-sucrose, 6 mM-MgCl₂, 1 mM-dithiothreitol, 0.04 mM each of dATP, dGTP, dCTP, 1 mM-ATP, 5 mM-PEP, 15 μg/ml PK, 5 mM-EDTA, 0.04 mM each of ATP, CTP, GTP, 1 mM-PHMB, 10 mM-(NH₄)₂SO₄, in 80 mM-potassium phosphate buffer in the presence of (a) 0.002 mM-α-UTP and 0.0087 mM-3H-UTP; (b) 0.002 mM-3H-TTP and 0.04 mM-α-UTP. ○—○, Untreated; O——O, in the presence of 0.4 μg/ml α-amanitin; ▲—▲, in the presence of 100 μg/ml α-amanitin.

addition of EGTA (Winnacker, Magnusson & Reichard, 1972). Addition of EGTA to HSV-infected nuclei after preparation in the presence of Ca²⁺, had no effect on DNA synthesis (not shown). These experiments revealed that calcium ions are neither required during the preparation of nuclei nor during the in vitro reaction.

Ca²⁺ only had an effect on in vitro DNA synthesis in the presence of the ATP generating system (ATP, PEP and PK; Fig. 1, curves 4 and 6). Under these conditions, in vitro DNA synthesis in the isolated nuclei was markedly enhanced and increased threefold. However, in the presence of Ca²⁺, degradation of the in vitro synthesized DNA was quite marked.

Effect of ATP generating system

ATP was reported to be necessary for initiating the formation of the enzymatic complex required for the synthesis of prokaryotic DNA in vitro (Wickner & Kornberg, 1973). Similarly, DNA synthesis in vitro in nuclei isolated from EBV-transformed lymphoblasts was stimulated by the presence of an ATP generating system in the reaction mixture (Benz & Strominger, 1975; Y. Becker, E. Weinberg & Y. Cohen, unpublished data). Addition of ATP, phosphoenolpyruvate (PEP) and phosphokinase (PK) to nuclei from HSV-infected cells resulted in a twofold increase in ³H-TMP incorporation (Fig. 1, curve 3) as compared to the control (Fig. 1, curve 1). Addition of the ATP generating system to nuclei prepared in the presence of Ca²⁺ (Fig. 1, curve 4) stimulated in vitro DNA synthesis but the rate of DNA synthesis was maximal after 30 min and was followed by degradation of the newly-synthesized DNA. These results indicated that the in vitro synthesis of DNA in nuclei from HSV-infected cells is stimulated by the addition of an ATP generating system. Preparation of the nuclei in the presence of Ca²⁺ seems to stimulate the nucleases which degrade the newly-synthesized DNA.
HSV-DNA synthesis in isolated nuclei

Fig. 3. Effect of (NH₄)⁺, PHMB and TTP on in vitro DNA synthesis. HSV-infected BSC 1 monolayers were scraped into RSB at 13 h p.i. and nuclei were prepared as described in Methods. Each 600 µl of reaction mixture contained 8 x 10⁶ nuclei in 80 mM-potassium phosphate buffer, pH 7.4, 250 mM-sucrose, 6 mM-MgCl₂, 0.4 mM-CaCl₂, 1 mM-dithiothreitol, 0.04 mM each of dATP, dGTP, dCTP and 0.02 mM of ³H-TTP, 1 mM-ATP, 5 mM-PEP, 15 µg/ml PK, 5 mM-EGTA and 0.04 mM each of ATP, CTP, UTP. The complete reaction mixture (O---O, curve 1) contained 1 mM-PHMB (C---C, curve 2) or 10 mM-(NH₄)₂SO₄ (△---△, curve 3); or 1 mM-PHMB and 10 mM-(NH₄)₂SO₄ (□---□, curve 4), or 0.013 mM-TTP (■---■, curve 5) or 0.013 mM-TTP and 1 mM-PHMB (□---□, curve 6). The mixtures were incubated at 37°C. Samples were withdrawn at the indicated time intervals and the TCA precipitable radioactivity in each sample was determined.

In all subsequent experiments, infected nuclei were prepared in phosphate buffer in the absence of Ca²⁺. The reaction mixtures contained EGTA, ATP and an ATP generating system (Fig. 1, curve 3) in addition to the four deoxyribonucleoside triphosphates.

RNA synthesis under conditions of DNA synthesis in vitro

Studies on the synthesis of SV₄₀ DNA in isolated nuclei in vitro, revealed the formation of RNA chains covalently bound to the nascent DNA chains (Reichard, Eliasson & Soderman, 1974). These RNA sequences served as primers for the synthesis of virus DNA. It was
therefore important to know whether in nuclei isolated from HSV-infected cells, RNA synthesis takes place under the conditions selected for in vitro DNA synthesis. Addition of ATP, CTP, GTP and \(^{3}H\)-UTP to HSV-infected nuclei only slightly stimulated DNA synthesis (Fig. 1, curves 5 and 6) as compared to the corresponding reaction mixtures that lacked the four ribonucleoside triphosphates (Fig. 1, curves 3 and 4).

It was also found (Fig. 2a) that \(^{3}H\)-UMP was incorporated into RNA chains both in the absence and presence of \(\alpha\)-amanitin. The incorporation of \(^{3}H\)-UMP was partially suppressed by \(\alpha\)-amanitin at the concentrations that inhibit the cellular RNA polymerases II and III (0.4 \(\mu\)g/ml and 100 \(\mu\)g/ml, respectively: Weinman, Raskas & Roeder, 1974; Ben-Zeev, Asher & Becker, 1976). These experiments show that RNA polymerase activities are detectable in vitro under conditions for DNA synthesis when the four ribonucleoside triphosphates are present.

The effect of \(\alpha\)-amanitin on DNA synthesis in vitro was investigated. Inhibition of the RNA polymerases II and III by 100 \(\mu\)g/ml of \(\alpha\)-amanitin was accompanied by a stimulation of in vitro DNA synthesis (Fig. 2b). The nature of this phenomenon is not known.

It has been shown (Ben-Zeev, Asher & Becker, 1976; A. Ben-Zeev & Y. Becker, to be published) that in HSV-infected nuclei in vitro, 10 mM-(NH\(_{4}\))\(_{2}\)SO\(_{4}\) selectively stimulates the activity of the cellular RNA polymerase II to transcribe HSV DNA. The effect of (NH\(_{4}\))\(_{2}\)SO\(_{4}\) was studied under the in vitro conditions selected for DNA synthesis. Addition of (NH\(_{4}\))\(^{+}\) to the reaction mixture had no effect on DNA synthesis (Fig. 3, curves 1 and 3).

**Effect of \(p\)-hydroxymercuribenzoate (PHMB)**

It was reported (Bohn, Matsukage & Wilson, 1974) that PHMB in the presence of dithiothreitol stimulated the synthesis of DNA in vitro by a mechanism that was not elucidated. Since the aim of the present study was to find in vitro conditions that would prolong DNA synthesis in the isolated nuclei, the effect of PHMB was studied. These experiments revealed that DNA synthesis was not only stimulated (Fig. 3, curve 2), as compared to DNA synthesis in the complete reaction mixture without PHMB (Fig. 3, curve 1), but was also prolonged from 60 min to 150 min. It was also found that PHMB in the presence of (NH\(_{4}\))\(_{2}\)SO\(_{4}\) extended the period of in vitro synthesis of DNA to 240 min, the maximum time tested (Fig. 3, curve 4).

As compared to the control (Fig. 3, curve 1) DNA synthesis was slower in the presence of PHMB (Fig. 3, curve 2) during the initial 60 min, but continued after DNA synthesis in the control had ceased. The in vitro synthesized DNA was partially degraded under these conditions but addition of (NH\(_{4}\))\(_{2}\)SO\(_{4}\) prevented the degradation of the in vitro product (Fig. 3, curve 4). Thus, the presence of PHMB is mandatory for prolonged in vitro DNA synthesis.

**Effect of thymidine triphosphate (TTP)**

Since \(^{3}H\)-TTP was used in the reaction mixture at a level of 0.002 \(\mu\)M, an experiment was performed in which, in addition to \(^{3}H\)-TTP, unlabelled TTP was added at the same concentration as the other three deoxyribonucleoside triphosphates (0.04 \(\mu\)M of each). This was done to determine whether the addition of excess TTP would affect the kinetics of DNA synthesis in vitro. Addition of TTP to the reaction mixture led to dilution and a lower extent of incorporation of \(^{3}H\)-TMP (Fig. 3, curves 5 and 6). DNA synthesis continued for a period of 60 min (Fig. 3, curve 5) while in the presence of PHMB (Fig. 3, curve 6), DNA synthesis was prolonged to 180 min. It is possible to conclude that an excess of TTP does not change the course of the in vitro DNA synthesis.
HSV-DNA synthesis in isolated nuclei

Fig. 4. Isolation of in vitro synthesized DNA in CsCl density gradients. Nuclei were prepared from HSV infected BSC 1 cells at 14 h p.i. as described in Methods. 2 x 10^6 nuclei were incubated at 37°C in 150 μl standard reaction mixtures that included ATP, PEP, PK, the four ribonucleoside triphosphates, PHMB and ammonium sulphate. After 15 min (a), 45 min (b), and 120 min (c) the reaction mixtures were adjusted to a final concentration of 1 x SSC; 0.5 mg/ml Pronase and 0.1% SDS were added followed by incubation at 37°C for 4 h. CsCl was added to bring the density of the solution to 1.68 g/ml in a vol. of 8.0 ml TE buffer, and the solutions were centrifuged in polyallomer tubes in the Ti 50 rotor of the Beckman preparative ultracentrifuge at 35 000 rev/min at 20°C for 44 h. The gradients were collected dropwise from the bottom of the tube and the TCA precipitable radioactivity in each fraction was determined. •-•, 3H-TMP incorporation into reaction product; ○-○, 14C-HSV DNA internal marker.
Characterization of the in vitro synthesized DNA

The properties of the DNA molecules synthesized in the nuclei isolated from HSV infected cells under the improved in vitro conditions with PHMB (Fig. 3, curve 4) were studied. The in vitro synthesized DNA was isolated by sedimentation in CsCl density gradients using HSV DNA from purified virions as a marker (Fig. 4). Most of the in vitro synthesized DNA banded at the density of HSV DNA (1.718 g/ml) as previously reported (Becker & Asher, 1975; Shlomai & Becker, 1975).

The size of the in vitro synthesized virus DNA, which was carefully separated from the cellular DNA by centrifugation in CsCl gradients, was studied by sedimentation in neutral sucrose gradients (Fig. 5a to c). Some of the virus DNA molecules that were labelled in vitro during the initial 15 min of incubation co-sedimented with the mature HSV DNA marker (mol. wt. 100 x 10^6; Becker, Dym & Sarov, 1968; Shlomai, Friedmann & Becker, 1976a). However, most of the labelled virus DNA molecules sedimented more slowly in the sucrose gradient (Fig. 6a). The labelled virus DNA isolated from nuclei which were incubated for a 30 min period (Fig. 5b) had sedimentation properties that differed from those of the intact HSV DNA marker. The virus DNA formed a distinct band with a sedimentation coefficient of about 50S. On further incubation of the nuclei in vitro, the labelled virus DNA product became much smaller and sedimented in the sucrose gradient with a sedimentation coefficient of about 25S or less (Fig. 5c). These results indicate that the synthesis of virus DNA in isolated nuclei may be accompanied by an extensive fragmentation of the virus DNA as
Comparatively shorter and shorter virus DNA fragments are synthesized in the isolated nuclei under the *in vitro* conditions.

To determine the size of the nascent virus DNA chains synthesized in isolated nuclei under *in vitro* conditions it was necessary to denature the isolated double-stranded virus DNA molecules into labelled single-stranded DNA chains. The virus DNA, after isolation in CsCl gradients was centrifuged in alkaline sucrose gradients (Fig. 6a to c) and the distribution of the labelled DNA chains was determined. The *in vitro* synthesized HSV DNA was found to consist of short DNA chains which banded close to the top of the sucrose gradient. A small fraction of the DNA chains synthesized *in vitro* during the initial 15 min (Fig. 6a) co-sedimented with the intact single-stranded molecules of the T2- and HSV-DNA markers (Fig. 6d). This result indicates that during the initial 15 min of *in vitro* incubation, completion of pre-existing DNA chains may occur. At later stages of *in vitro* incubation, only short DNA chains are synthesized.

**DISCUSSION**

The present study deals with the requirements of nuclei isolated from HSV infected cells for the synthesis of virus DNA under *in vitro* conditions. The infected nuclei were incubated in a reaction mixture prepared in phosphate buffer which inhibited DNA synthesis *in vitro* in uninfected nuclei (Becker & Asher, 1975). HEPES buffer (Kolber, 1975; Y. Becker & Y. Asher, unpublished results) supported DNA synthesis in HSV-infected nuclei for a 20 min period, followed by degradation of the *in vitro* synthesized DNA. In contrast, phosphate buffer allowed DNA synthesis to continue for 60 min and only some of the *in vitro* labelled DNA molecules were degraded on further incubation. In an attempt to improve the conditions for *in vitro* DNA synthesis we tested the effects of Ca$^{2+}$, ATP and an ATP generating
system, a sulphhydryl compound (PHMB) and RNA synthesis on virus DNA synthesis in the isolated nuclei. Although conditions that permit the unaffected semiconservative replication of virus DNA in isolated nuclei in vitro were not obtained, the present study provides basic information on the conditions for in vitro DNA synthesis.

The presence of Ca²⁺ in the buffer used for the preparation of nuclei from SV40-infected cells (Magnusson et al. 1972, 1973; Otto & Reichard, 1975; De Pamphilis & Berg, 1975; De Pamphilis, Beard & Berg, 1975) was reported by Qasba (1974a,b) to be essential for obtaining unaffected SV40 DNA synthesis in vitro. In our previous studies (Becker & Asher, 1975; Shlomai & Becker, 1975) Ca²⁺ was not used and DNA synthesis in vitro was demonstrable for a 60 min period. In the present study, preparation of nuclei in the presence of Ca²⁺ only slightly affected DNA synthesis in the absence of ATP and an ATP generating system. However, in the presence of an ATP generating system, which stimulated in vitro DNA synthesis, preparation of nuclei in the presence of Ca²⁺ led to a marked degradation of the in vitro synthesized DNA (Fig. 1, curves 4 and 6). This suggests that Ca²⁺ stabilizes the DNases present in the nuclei, leading to degradation of the in vitro synthesized DNA. In the absence of Ca²⁺ the in vitro-synthesized DNA is quite stable.

In the presence of ATP and an ATP generating system, 350000 ct/min of ³H-thymidine monophosphate (175 pmol) were incorporated by approx. 2 x 10⁶ nuclei, indicating an improvement in the in vitro conditions for HSV DNA synthesis in isolated nuclei. The requirement for ATP also indicates semiconservative synthesis of the virus DNA since similar conditions were required for the in vitro synthesis of prokaryotic DNA (Wickner & Kornberg, 1973).

Addition of the four ribonucleoside triphosphates was not found to be necessary for DNA synthesis. It is not yet known whether initiation of virus DNA synthesis occurs under our in vitro conditions in the isolated nuclei. The lack of a requirement for ribonucleoside triphosphates does not rule out the possibility of initiation of DNA chains with RNA in isolated nuclei, as reported in nuclei from SV40-infected cells (Eliasson, Martin & Reichard, 1974; Piglet, Eliasson & Reichard, 1974; Reichard et al. 1974).

The in vitro synthesis of DNA in HSV-infected nuclei was markedly stimulated by the addition of PHMB, a sulphhydryl reagent. Under these conditions the synthesis of DNA was prolonged for 4 h in the presence of (NH₄)⁺ without degradation of the newly synthesized DNA (Fig. 3). In this respect HSV-infected nuclei differ from SV40-infected nuclei in that DNA synthesis was inhibited when p-chloromercurobenzoate (PCMB) was added to the appropriate SV40 reaction mixture (de Pamphilis & Berg, 1975). These observations may indicate that the HSV-coded DNA polymerase differs from that which replicates SV40 DNA.

It is of interest that the addition of (NH₄)⁺ to the reaction mixture improved DNA synthesis in the presence but not in the absence of PHMB. Under in vitro conditions for RNA synthesis in infected nuclei (A. Ben-Zeev & Y. Becker, to be published) 10 mM (NH₄)₂SO₄ was shown to stimulate HSV DNA transcription by the cellular RNA polymerase II. It may be that under in vitro conditions for DNA synthesis, (NH₄)₂SO₄ stimulates HSV DNA transcription only in the presence of PHMB.

Our initial study (Becker & Asher, 1975) on in vitro DNA synthesis in nuclei isolated from HSV-infected cells showed by DNA-DNA hybridization (Cedar, 1976) that most (about 90%) of the synthesized DNA molecules were of virus specificity. The analysis of the newly synthesized DNA by centrifugation in alkaline sucrose gradients revealed labelled single-stranded DNA fragments close to the top of the gradient. Similarly, centrifugation in a neutral sucrose gradient revealed that during the initial 15 min of incubation in vitro, some
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radioactive DNA molecules co-sedimented with the HSV DNA marker as mature (100 × 10^6 daltons) virus DNA molecules, while the rest of the radioactive DNA banded between the mature virus DNA and the top of the gradient in a heterogeneous band. The conclusion from these experiments was that the replicating DNA molecules which were close to being completed when the nuclei were transferred to \textit{in vitro} conditions gave rise to the mature labelled virus DNA molecules. It may also be possible that HSV DNA is synthesized as short single-stranded chains, similar to those reported for adenovirus (van der Vliet & Sussenbach, 1972) and SV40 (Fareed, Khoury & Salzman, 1973).

Recent studies on the replicative intermediates of HSV DNA (Shlomai et al. 1976a, b; A. Friedmann, Y. Shlomai & Y. Becker, to be published) revealed that molecules having replicative loops or a Y-shape are the templates for DNA synthesis. We assume that these same DNA molecules are the templates for virus DNA synthesis in isolated nuclei \textit{in vitro}. However, the fact that the HSV DNA molecules synthesized \textit{in vitro} sediment in sucrose gradients more slowly than found with the \textit{in vitro} system in BSC-1 cells may indicate that nucleases are active in the isolated nuclei. Elucidation of conditions that would permit unaffected semiconservative synthesis of virus DNA is still needed.

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