Cell-Free Synthesis of Herpes Simplex Virus DNA: The Influence of Polyamines†

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ABSTRACT: The effect of polyamines on cell-free DNA synthesis of herpes simplex virus DNA in two different systems is investigated. Purified nuclei from infected cells are devoid of measurable amounts of putrescine, spermidine, and spermine, while an unfractionated lysate contains the polyamines at close to their respective cellular concentrations. Spermine, 0.3 mM, and 0.5 mM spermidine, when added to the nuclear system, decrease the extent of viral DNA synthesis to the level found in the lysate system, the size of the cell-free viral DNA product is increased, and a specific inhibition of repair-type DNA synthesis is observed. These effects of the polyamines occur only in the presence of ATP and not the other three ribonucleoside triphosphates.

To analyze the mechanism of herpes simplex virus (HSV)[1] DNA replication, isolated nuclei from infected cells have been used for cell-free synthesis of viral DNA (Kolber, 1975; Becker & Asher, 1975; Bolden et al., 1975; Biswal & Murray, 1974). Several features of the synthesis of viral DNA in isolated nuclei make it doubtful that it resembles replicative synthesis in infected cells: the size of the in vitro product is small, extensive repair type synthesis takes place, and the in vitro reaction does not require ATP. A different cell-free system (Francke, 1977a), consisting of an unfractionated hypotonic lysate from infected cells, is capable of synthesizing a larger size in vitro product. When comparing the lysate system with the isolated nuclear system, the following observations were made (Francke, 1977b): for HSV, maximal in vitro synthesis rates do not require the presence of cytoplasmic factors, which is in contrast to papovavirus-derived systems (Francke & Hunter, 1975), but similar to in vitro adenovirus DNA synthesis (Yamashita et al., 1977); purified nuclei incorporate labeled deoxynucleoside triphosphates into HSV DNA for longer times than the lysate, but the size of in vitro labeled and preexisting viral DNA is small, due to endonucleolytic cleavage during the in vitro incubation. It appears, therefore, that the purified nuclear system is lacking factors which protect viral DNA from nucleases during its replication in infected cells or in the unfractionated lysate, leading to the "activation" of normally inactive viral DNA templates for in vitro synthesis. In contrast to cellular DNA, HSV DNA is not contained in a chromatin-like structure during infection (Francke, 1977b). The protection of the viral DNA from endonuclease in the lysate, therefore, is most likely due to the presence of factors which are removed from the nuclei during the purification. Since polyamines (specifically spermine) have been shown to be complexed to the viral DNA in the HSV virion (Gibson & Roizman, 1971), it is possible that they might also play a role in stabilizing the viral DNA during replication in the nucleus of infected cells. The study presented here was undertaken to test this possibility for cell-free HSV DNA synthesis in purified nuclei.

Experimental Procedures

Cells and Virus. BHK C13 cells (MacPhearson & Stocker, 1962) and wild-type HSV-1 syn$^+$ (Glasgow strain 17) were used throughout this study. Virus was passaged and titered as described (Francke, 1977a). For experimental purposes, cells were infected at a moi of 10 pfu/cell, and cell-free DNA synthesis systems were prepared at 11 h after infection at 31 °C.

Polyamine Determination. Nine centimeter dishes, containing 2×10^7 uninfected or infected cells, were rinsed with isotonic Tris buffer. After addition of 4 mL of 5% trichloroacetic acid, the contents of the dish was scraped into a centrifuge tube. Cell-free preparations were precipitated directly. The precipitate was removed by two centrifugations at 4500*g* and 4 °C and the supernatant was stored at −20 °C. The contents of spermine, spermidine, and putrescine were determined using an amino acid analyzer (Kremzer, 1973) after prior calibration with the respective standards of known concentration. Results are presented in mM concentrations, calculated for a volume of 150 μL, routinely obtained for a concentrated hypotonic lysate from one 9-cm dish containing 2×10^7 cells, assuming the same volume for intact cells.

Viral Marker DNAs. [^{3}H]dThd-labeled viral capsids were prepared as described (Francke, 1977b). Normal density ^{32}P-labeled DNA (LL) was prepared similarly after labeling infected cells in low phosphate medium (2×10^{-5} M NaH_2PO_4) in the presence of 2% dialyzed calf serum with 100 μCi carrier-free [^{32}P]/mL for 20 h. For BrdU substituted DNA (HH), bromodeoxyuridine (20 μg/mL), fluorodeoxyuridine (5 μg/mL), and cytidine (12 μg/mL) were present in addition. The labeled capsids were added at the time of termination of in vitro reactions.

Cell-Free DNA Synthesis. Hypotonic buffer contained 20 mM Hepes (adjusted to pH 7.9 with KOH), 1 mM $MgCl_2$, 1 mM DTT. Isotonic buffer contained in addition 0.25 M sucrose, 20 mM Hepes, pH 7.9, and 80 mM potassium acetate. Washing buffer for nuclei was the same as isotonic buffer, except that the potassium acetate was replaced with KCl. Lysate, nuclei, and cytosol were prepared as described (Francke, 1977a). Nuclei were routinely washed twice in

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[1] Abbreviations used: HSV, herpes simplex virus; pfu, plaque-forming units; moi, multiplicity of infection; dThd, thymidine; BrdU, bromodeoxyuridine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DDT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetate.

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TABLE I: Polyamine Concentrations in BHK Cells at Various Times after Infection with HSV.[a]

hours after infection at 31 °C	mM[b] putrescine	spermidine	spermine
0	0.021	0.492	0.510
6	0.090	0.456	0.475
12	0.174	0.660	0.463
18	0.306	0.650	0.500

[a] Multiplicity of infection: 10 plaque-forming units/cell. [b] mM concentration in cell volume (2×10^7 cells in 150 μL).

TABLE II: Polyamine Concentrations in HSV-Infected BHK Cells and Subcellular Fraction Derived from Them.[a]

	mM[b] putrescine	spermidine	spermine
cells	0.164	0.471	0.327
lysate	0.101	0.348	0.261
nuclei	<0.001[c]	<0.005[c]	<0.005[c]
cytosol	0.079	0.125	0.063

[a] Twelve hours infection at 31 °C with 10 plaque-forming units/cell. [b] mM concentration (2×10^7 cells, or cell equivalents, in 150 μL). [c] Upper limit of detectability.

washing buffer and finally resuspended in isotonic buffer corresponding to the volume of the lysate. Polyamines, when added, were present as of the second wash. Later addition gave less reproducible results. Prior to preparation of the system, infected cells were prelabeled with [^{3}H]dThd (13.4 Ci/mmol, 5 μCi/mL, in 5 mL of medium/9 cm dish) for 1 h at 31 °C. For cell-free DNA synthesis, the following additions were present (final concentrations, including the components of the buffers): 2 mM ATP; 100 μM each of CTP, GTP, UTP, dCTP, dGTP, TTP; 20 μM dATP; 1 mM DTT; 1 mM EDTA; 40 mM Hepes, pH 7.9; 5 mM $MgCl_2$; 80 mM potassium acetate; and [α-^{32}P]dATP (130 Ci/mmol, 100 μCi/mL). Reactions were carried out at 31 °C and terminated as described (Francke, 1977a). For density labeling experiments, the TTP was omitted and replaced by 200 μM BrdUTP. This has no effect on the rate or extent of in vitro labeling with [α-^{32}P]dATP.

Centrifugation Techniques. Separation of viral from cellular DNA was by equilibrium centrifugation in CsCl gradients as described (Francke, 1977a). For preparative purposes, gradients were fractionated and aliquots assayed for radioactivity. Fractions corresponding to viral DNA were dialyzed against two changes of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl. Sedimentation analyses were in 5–20% w/v sucrose gradients in 1 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 8.0), and 0.1% sarcosyl. Gradient, 3.2 mL, was layered over a 0.5-mL cushion of saturated CsCl in 20% sucrose. Samples, 150 μL, were applied to the top of the gradient and centrifuged in a Beckman SW 56 rotor at 45 000 rpm, and 20 °C, for 90 min. Fractions (21 ± 1) were collected from the bottom of the tube and processed as described (Francke, 1977a). For BrdU-substituted DNA, CsCl density gradients were adjusted to an initial density of 1.740 g/cm^3 and centrifuged for 60 h at 38 000 rpm in a Beckman 50 Ti rotor. Under these conditions, cellular DNA banded at a density of 1.705 g/cm^3, unsubstituted viral DNA at 1.730 g/cm^3, and viral DNA made in vivo in the presence of BrdU at 1.787 g/cm^3, as determined by the refractive index of individual fractions after collection of the gradient (compare Figure 4).

Results

The concentrations of putrescine, spermidine, and spermine in BHK cells at various times after infection with HSV (Table I) indicate no change for spermine and a small increase for spermidine. The large increase in putrescine, particularly late in infection, is likely to be a consequence of virus mediated turnoff of host protein synthesis resulting in a lack of the cellular enzymes, which normally process putrescine to the higher polyamines (Williams-Ashman et al., 1973). The virus does not appear to induce increased synthesis of the higher polyamines. As shown in Table II, an unfractionated hypotonic lysate of infected cells contains similar amounts of polyamines as the intact cells. The loss of ca. 30% of each of the three compounds measured may have occurred during the hypotonic swelling. After fractionation of the lysate, ca. 50% of the spermidine and ca. 25% of the spermine appear in the cytosol, the rest presumably being bound to the pellet of cytoplasmic microsomes, while the nuclei contain no measurable polyamines. The numbers given for nuclei in Table II, representing the upper limit of detectability, are in the μM range, where no effect on cell-free DNA synthesis is observed (see below). The lack of polyamines in purified nuclei is in contrast to observations by others (Gibson & Roizman, 1973), and is likely to be due to the different washing procedures. The washing procedure used in this study is essential for nuclear preparations with sufficiently low levels of exonuclease compatible with cell-free DNA synthesis (Francke, 1977b).

The experimental system to test the influence of added polyamines on cell-free DNA synthesis for all experiments reported here was as follows: infected cells were prelabeled in vivo for 1 h with [^{3}H]dThd, and nuclei and lysate were prepared in parallel and kept at identical concentration (2×10^7 nuclei/150 μL); in vitro incubation was in the presence of [α-^{32}P]dATP at 20 μM to avoid the contribution of changing deoxynucleotide pools (Francke, 1977a) to the specific radioactivity of the precursor. The fate of the ^{3}H label provides a measure of exonucleolytic activity, of which even highly purified nuclei are not totally devoid (Francke, 1977b and Figure 3b), while the ^{32}P label monitors in vitro DNA synthesis. The experiment shown in Figure 1 shows such an experiment for purified nuclei in the presence of various concentrations of spermine, spermidine, and putrescine, including Mg^{2+} as a control. The product consisted of >80% viral DNA (as determined by CsCl density gradient centrifugation), and the extent of synthesis in the lysate system was 3.4×10^4 ^{32}P cpm per 120 min per 5×10^6 nuclei (not included in the figure). In the absence of ATP (Figure 1a), spermine inhibits the extent of synthesis strongly, spermidine less so, while putrescine has no effect beyond that of Mg^{2+}. The time courses of in vitro synthesis (not shown, compare also Figure 3) indicated that the inhibition was mainly of the extent with no effect on the initial rate of synthesis (up to 0.5 mM for spermine, and 1.5 mM for spermidine). The differences in inhibition by spermine over spermidine, and by the two higher polyamines compared with putrescine are apparent not only on the basis of molar concentration (as plotted in the figure), but even if the difference in valency between the three polyamines is taken into account. When evaluating the stability of prelabeled DNA (^{3}H cpm in Figure 1), spermine is most effective at protecting the DNA from exonuclease(s), while spermidine and putrescine do not increase the stability of the prelabel, at least up to 1.5 mM. The stability of the prelabel does not exclude endonucleolytic cleavage, though, as long as the product remains acid precipitable (see below). In purified nuclei, complete inhibition of synthesis (>95%) and stability of prelabel can be achieved

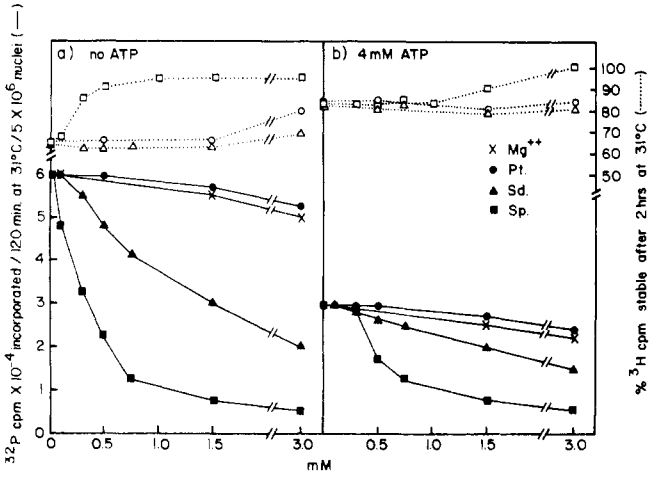

FIGURE 1: Influence of Mg^{2+} and polyamines on the synthesis and stability of viral DNA during incubation in purified nuclei. BHK cells were infected with HSV and prelabeled with [^{3}H]dThd from 10 to 11 h after infection and nuclei were prepared as described in Experimental Procedures. $MgCl_2$, putrescine (Pt), spermidine (Sd), and spermine (Sp) were added at the concentrations indicated. For each point, a 200-μL reaction was carried out under standard conditions (see Experimental Procedures) with the following modifications: ATP was omitted and the $MgCl_2$ reduced to 3 mM (panel a), or ATP was present at 4 mM and $MgCl_2$ increased to 7 mM (panel b). The mM concentrations indicated for Mg^{2+} in the figure are in addition to these. Samples (40 μL) were removed at 0, 10, 30, 60, and 120 min after the start of the reaction at 31 °C, and the acid-precipitable ^{3}H and ^{32}P radioactivities were determined. The results for the 120-min points are presented. DNA synthesis ([^{32}P]cpm, solid symbols), at various concentrations of $MgCl_2$ (X), putrescine (●), spermidine (▲), or spermine (■), in the absence (a) or presence (b) of 4 mM ATP.

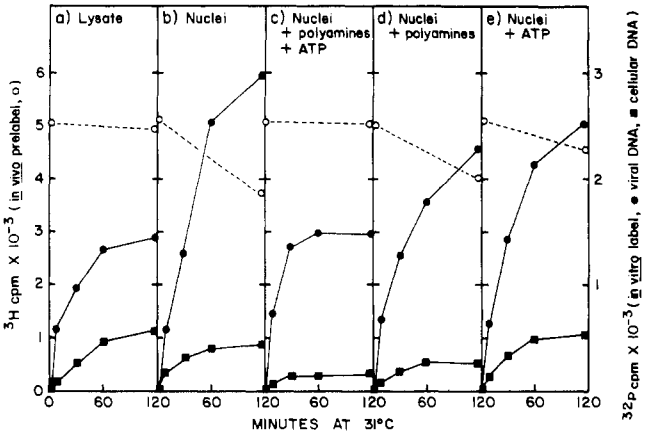

FIGURE 2: Viral and cellular DNA synthesis in the lysate and in purified nuclei in the presence and absence of polyamines and ATP. Cells were infected and prelabeled with [^{3}H]dThd as described in Experimental Procedures. Eight milliliters of hypotonic lysate was divided into 5 parts, and 4 parts were processed into nuclei with spermine (0.3 mM), spermidine (0.5 mM), and ATP (2 mM) as indicated. Each set was divided into two portions, and one was incubated under standard conditions, while in the other BrdUTP was substituted for TTP. Samples were removed at the times indicated and analyzed in CsCl equilibrium gradients. The results from the reactions containing TTP are shown in the figure. Prelabeled viral DNA (○, ^{3}H cpm), and in vitro labeled viral (●) and cellular (■) DNA ^{32}P cpm in the lysate (a), nuclei in the absence (b) or presence of ATP and polyamines (c), and with polyamines (d) or ATP (e) separately.

TABLE III: Effect of Ribonucleoside Triphosphates on DNA Synthesis and Degradation in Nuclei from HSV-Infected BHK Cells, in the Absence and Presence of Polyamines.[a]

additions[d] (2 mM)	% synthesis[b] (120 min, 31 °C) polyamines −	+	% stability[c] (120 min, 31 °C) polyamines −	+
none	183	138	72	78
$MgCl_2$	181	136	64	62
ATP	248	93	71	98
CTP	208	147	79	81
GTP	179	162	67	69
UTP	183	148	73	75

[a] Polyamines: 0.3 mM spermine and 0.5 mM spermidine. [b] 100% = 2.05×10^4 ^{32}P cpm, incorporated per 2×10^7 nuclei in the lysate system derived from the same set of infected cells incubated under standard conditions (Experimental Procedures) for 120 min. [c] 100% = 2.93×10^4 ^{3}H cpm, derived from a 1-h in vivo prelabel with [^{3}H]-dThd (Experimental Procedures), present at the start of the reaction. [d] 2 mM additional $MgCl_2$ was present in the reactions containing 2 mM ribonucleoside triphosphate.

with spermine (at 4.5 mM) and spermidine (at 6 mM) but not with putrescine (tested up to 12 mM, data not shown). In the presence of 4 mM ATP (Figure 1b), the inhibitory effects are less pronounced. As reported previously (Francke, 1977a), ATP by itself inhibits synthesis in purified nuclei above 2 mM. In a series of experiments, similar to those shown in Figure 1, the combination of 0.3 mM spermine, 0.5 mM spermidine, which is close to their respective concentrations in the lysate (Table II), and 2 mM ATP resulted in optimal stability of prelabeled DNA. The optimal $MgCl_2$ concentration for in vitro synthesis was unchanged (5 mM) by the presence of the polyamines. The extent of synthesis under these conditions was found to be inhibited to approximately that occurring in the control lysate. This combination was therefore used throughout the following experiments.

The experiment shown in Table III was performed in order to test whether in this concentration range the additive effects of ATP and the polyamines could also be achieved with the other ribonucleoside triphosphates. The incubations were carried out in the absence of all ribonucleoside triphosphates, except for the one added, and with a basic $MgCl_2$ concentration of 3 mM. In the absence of polyamines, neither the stability of the prelabel nor the extent of synthesis is significantly changed by any of the four triphosphates. In the presence of polyamines, the inhibition of synthesis and degradation appear to be specific for ATP, and ATP cannot be substituted for by CTP, GTP, or UTP. The beneficial effect of ATP, specifically in combination with spermine, became more obvious when the size of the viral DNA labeled in vitro was analyzed (data not shown). In the absence of polyamines, the small size of the product was unchanged by any combination of ribonucleoside triphosphates. In the presence of spermine, a significant increase in the average size of the product was caused by ATP, yet much less so by the combination of CTP, GTP, and UTP.

The final experiment (Figures 2–5) shows a comparison of synthesis in the lysate (a), nuclei in the absence (b) and presence (c) of polyamines and ATP, and in the presence of polyamines (d) or ATP (e), separately. After the in vivo prelabel, the in vitro reactions were carried out in duplicate, once under standard reaction conditions (Figures 2 and 3) and once by substituting BrdUTP for TTP (Figures 4 and 5). The BrdUTP substitution had no effect on total in vitro dATP incorporation in any of the systems (not shown). The products of the reactions in the presence of TTP were analyzed in preparative CsCl gradients and the time courses for in vitro labeling of viral and cellular DNA are shown in Figure 2. The excess of viral DNA labeled by nuclei (b) over that seen in the lysate (a) was reversed by the presence of ATP and polyamines (c), but not by

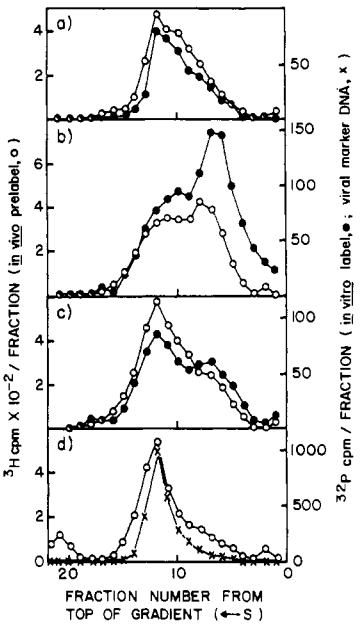

FIGURE 3: Sucrose gradient sedimentation of in vivo prelabeled and in vitro labeled viral DNA. Experimental details were as described in legend to Figure 2. The 60-min viral DNA products from the reactions containing TTP were purified in CsCl gradients and analyzed by sedimentation in sucrose gradients as described in Experimental Procedures. In vivo prelabel (O, ^{3}H cpm) and in vitro label (●, ^{32}P cpm) in viral DNA after 60 min of incubation in the lysate (a), purified nuclei in the absence (b) and presence of ATP and polyamines (c), and the profile of the in vivo prelabel (O) prior to in vitro incubation, cosedimented with ^{32}P-labeled viral marker DNA (X) (d).

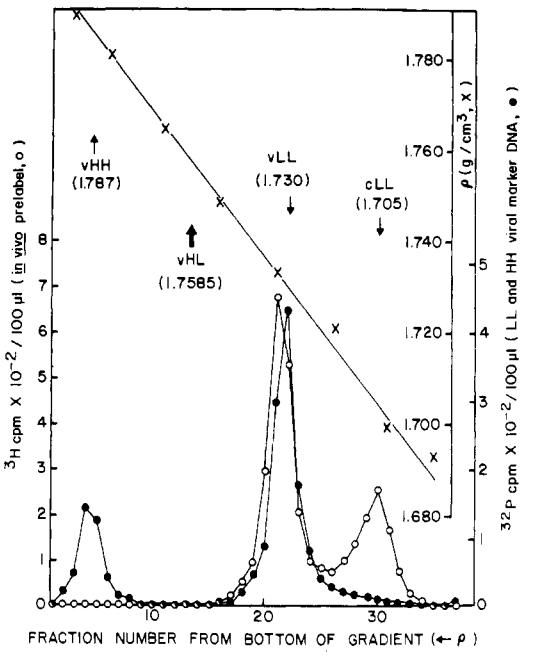

FIGURE 4: Profiles of viral markers and in vivo prelabeled DNA from infected cells in a CsCl equilibrium gradient. The zero time sample of the reactions containing BrdUTP (see legend to Figure 3) was mixed with viral capsids labeled with ^{32}P in vivo in the absence (LL) or presence (HH) of BrdU (see Experimental Procedures). The refractive index was determined immediately after collection of the gradient in the fractions indicated to determine the density. ^{32}P cpm (●, viral markers), ^{3}H cpm (O, in vivo prelabeled DNA), density (X, g/cm^3). The arrows indicate the position of unsubstituted cellular (cLL), unsubstituted viral (vLL), substituted viral (vHH), and the expected density for half-substituted viral DNA (vHL). Density increase was from right to left.

polyamines (d) or ATP alone (e). Similarly, the prelabeled viral DNA in the nuclei was stable only in the presence of polyamines and ATP. Prelabeled cellular DNA was stable under all conditions (not shown in the figure). The sedimentation analysis of the viral DNA is shown in Figure 3 for the 60-min in vitro reactions in the lysate (a), nuclei in the absence (b) and presence (c) of polyamines and ATP, and compared with the zero time sample in panel d, cosedimented with viral marker DNA. The distribution of the prelabel prior to in vitro incubation shows, in addition to unit length and some smaller DNA, structures slightly faster than mature DNA and rapidly sedimenting material, caught on the saturated CsCl cushion. Both of the faster sedimenting viral DNA species have been observed by others (Ben-Porat et al., 1976; Jacob & Roizman, 1977; Hirsch et al., 1977) and might represent replicating DNA. After 60 min of in vitro incubation in the lysate (a), a large proportion of the viral DNA, prelabeled and in vitro synthesized, is of genome size, while the product from isolated nuclei (b) shows evidence for extensive endonucleolytic cleavage, most evident for the in vitro label but also affecting the prelabeled DNA. The addition of ATP and polyamines to isolated nuclei (c) results in increased protection of the prelabeled DNA and a greater proportion of in vitro labeled DNA sedimenting at the rate of mature viral DNA. The profiles obtained for viral DNA in isolated nuclei with either ATP or the polyamines present separately (not shown) were similar to the profile in panel b. The densities of the products of the in vitro reactions carried out in the presence of BrdUTP were analyzed in CsCl equilibrium gradients. The distribution of the prelabel ^{3}H along with normal (LL) and BrdU substituted (HH) viral marker DNAs ^{32}P is shown in Figure 4. The slight shift toward higher density of the ^{3}H label is likely to be due to the single-stranded regions known to be present in replicating DNA (Schlomai et al., 1976). From the densities of the

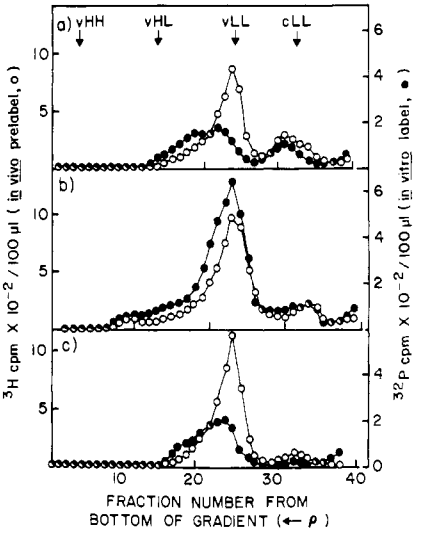

FIGURE 5: Density profiles of in vivo prelabeled and in vitro labeled DNA after cell-free synthesis in the presence of BrdUTP. Experimental details were as described in legend to Figure 2. The 60-min products of the reactions containing BrdUTP were analyzed in CsCl equilibrium gradients. The density of selected fractions was determined as described in legend to Figure 4, and was used to align the three panels shown in the figure. In vivo prelabel (O, ^{3}H) and in vitro label (●, ^{32}P) after 60 min of incubation in the lysate (a), or purified nuclei in the absence (b) or presence of polyamines and ATP (c).

viral HH and LL DNA peaks, a density of 1.7585 g/cm^3 can be predicted for half-substituted viral DNA (HL). The density profile of the 60-min product in the lysate system (Figure 5a) shows an increased density for a portion of the prelabel ^{3}H

indicating that in vitro synthesis utilizes preexisting templates. The in vitro label ^{32}P spans the density range between viral LL and the predicted HL positions. This distribution is compatible with semiconservative DNA synthesis on preexisting replicating DNA of various stages of completion, and suggests that reinitiation of molecules completed in vitro does not occur at detectable frequency. The density of the in vitro product in purified nuclei (Figure 5b) is predominantly that of LL DNA with some asymmetry toward higher density. This confirms an earlier observation (Kolber, 1975) that nuclei from HSV infected cells carry out extensive repair type DNA synthesis. The profile in Figure 5b does not exclude that some semiconservative synthesis occurs at the same time. The finding of a small amount of DNA at greater than HL density indicates the existence of product molecules with BrdU substitution in excess of one strand, which could have arisen secondary to nucleolytic events by self-priming or by replacement of the original template strand. The addition of polyamines and ATP to purified nuclei restored a density profile of the viral DNA product (Figure 5c), resembling that seen in the lysate. The relative amount of in vitro label banding at LL density was reduced by ATP and polyamines, indicating a preferential inhibition of repair type synthesis by these compounds. Again, the profiles of the products obtained with either ATP or the polyamines alone (not shown) were similar to that in Figure 5b, implying that the combination of both is required for the effect.

Discussion

The study presented here compares two types of cell-free DNA synthesis systems derived from HSV-infected cells. Because of the rather limited knowledge of the normal HSV DNA replication mechanism, the potential significance of the findings for in vivo viral DNA synthesis will not be discussed. But several features of the lysate system suggest that it might support viral DNA synthesis in a fashion resembling the in vivo process more closely than do purified nuclei: synthesis in the lysate is limited—compatible with completion of replicating molecules in the absence of initiation of new molecules—and up to genome size products are made by a predominantly semiconservative mechanism, while in the nuclear system repair type synthesis is predominant and complicated by extensive endonucleolytic and some exonucleolytic activities.

The nuclear system lacks endogenous polyamines, while the lysate contains spermine, spermidine, and putrescine at close to their respective cellular concentrations. Adding spermine, spermidine, and ATP to the nuclear system changes a number of features of the DNA synthesis, observed in nuclei, to resemble that occurring in the lysate: limited synthesis time, less endo- and exonucleolytic activity, larger product size, and less repair type synthesis. These effects are crucially dependent on the amount of added polyamines, and too high concentrations result in inhibition of DNA synthesis beyond that observed in the lysate. A strong argument for the potential relevance of the findings is that the optimal concentrations for the protection of the viral DNA with least inhibition of synthesis are close to the respective in vivo concentrations. Another point is the specific requirement for the presence of ATP for the beneficial effects of the polyamines to occur since, in most other in vitro systems (Wickner & Kornberg, 1973; Hunter & Francke, 1974; Alberts et al., 1975), ATP is required for DNA replication. Lastly, the observation that spermine is more effective than spermidine can be correlated with the known specific association of spermine with the viral DNA in the HSV virion (Gibson & Roizman, 1971). While such arguments suggest a specific involvement of spermine in HSV DNA replication, they do not exclude the possibility that other "cytoplasmic" factors are required in addition. During the purification by the methods employed, a large number of proteins and low molecular weight compounds are likely to be removed from the nuclei in addition to the polyamines. Experiments not reported in this communication have shown that polyamines added to the lysate at concentrations which inhibit DNA synthesis in isolated nuclei completely, have no effect on DNA synthesis in the lysate system. This illustrates that the situation in purified nuclei is rather different from the lysate with respect to the diffusibility of polyamines and/or their binding to structures other than viral DNA. But irrespective of their specific involvement or substitution for other factors removed from the nuclei, the addition of polyamines makes the nuclear system more suitable for studies concerning the detailed mechanism of HSV DNA replication.

The differential effect of the polyamines compared with Mg^{2+} and the unchanged Mg^{2+} optimum in the presence of polyamines indicate that the observed effects are specific for the two higher polyamines and do not simply substitute for Mg^{2+}. Since viral DNA replication probably involves a complex of several proteins and DNA, it cannot be decided at this time through which of these structures the polyamines act primarily. They might be required for the stability of the viral DNA by direct binding or indirectly by interacting with DNA binding proteins (Christiansen & Baldwin, 1977). Alternatively, they may play a role for the activity of individual enzymes, such as the polymerase, or directly inhibit degradative enzymes, such as nucleases or ATPases. It should be mentioned that, for cell-free synthesis of Epstein-Barr virus DNA in isolated nuclei (Seebeck et al., 1977), similar polyamine additions have been made as reported here.

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Collagen Synthesis by Human Amniotic Fluid Cells in Culture: Characterization of a Procollagen with Three Identical Proα1(I) Chains†

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ABSTRACT: Second trimester human amniotic fluid cells synthesize and secrete a variety of collagenous proteins in culture. F cells (amniotic fluid fibroblasts) are the most active biosynthetically and synthesize predominantly type I with smaller amounts of type III procollagen. Epithelioid AF cells (the predominating clonable cell type) synthesize a type IV-like procollagen and a procollagen with three identical proα chains, structurally and immunologically related to the proα1 chains of type I procollagen. The latter procollagen, when cleaved with pepsin and denatured, yields a single non-disulfide-bonded α chain that migrates more slowly than F cell or human skin α1(I) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis but coelutes with these chains from carboxymethylcellulose. The major cyanogen bromide produced peptides demonstrate a similar behavior relative to peptides derived from α1(I). The collagen is characterized by an increased solubility at neutral pH and high ionic strength, relative to type I collagen. The amino acid composition of the pepsin-resistant α chain is essentially identical with that of human α1(I), except for marked increases in the content of 3- and 4-hydroxyproline and hydroxylysine. Preliminary experiments suggest that these increased posttranslational modifications are responsible for the unusually slow migration of this collagen and its cyanogen bromide peptides on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The procollagen has, therefore, been assigned the chain composition [proα1(I)]$_3$. Like type I procollagen, [proα1(I)]$_3$ undergoes a time-dependent conversion, in the medium and cell layer, to procollagen intermediates and α chains. The production of [proα1(I)]$_3$ probably reflects the state of differentiation and/or embryologic derivation of AF cells rather than a characteristic of the fetal phenotype, since F cells do not synthesize significant amounts of the procollagen.

Fetal cells cultured from amniotic fluid have been utilized for the prenatal diagnosis of a variety of cytogenetic and inherited metabolic disorders (Milunsky, 1973). There has, however, been confusion in the literature regarding the morphologic identification of amniotic fluid cells and very little is known about their embryologic origin or state of differentiation. Although the presence of epithelioid cells in amniotic fluid has been noted, the cells emerging from long-term mass cultures were most frequently described as fibroblasts or fibroblast-like. Recent studies by Hoehn et al. (1974, 1975) have demonstrated that there are at least three distinct cell types which may be cloned from second trimester human amniotic fluid and subsequently propagated in culture. The cells were designated as E (epithelial), F (fibroblastic), and AF (cells with intermediate morphology and the predominating clonable cell type). These cell types have been distinguished on the basis of their clonal and cellular morphology (Hoehn et al., 1974, 1975), ultrastructure, growth characteristics (Hoehn et al., 1974), and most recently by their secretory products (Priest et al., 1977; Megaw et al., 1977; Crouch et al., 1978).

Relatively little is known about collagen production by amniotic fluid cells. Macek et al. (1973) presented evidence that mass cultures of cells with "uniform fibroblast morphology" synthesized hydroxyproline-containing proteins. Subsequently, Hurych et al. (1976) demonstrated the synthesis of type I collagen by long-term cultures of "amniotic fluid fibroblasts". Most recently, Priest et al. (1977) examined the collagens synthesized by clones and mass cultures of F and AF cells. F cells were found to synthesize type I collagen on the basis of chromatography on CM[1]-cellulose. However, AF cells

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[1] Abbreviations used: DMEM, Dulbecco-Vogt modified Eagle's medium; FCS, fetal calf serum; PBS, calcium- and magnesium-free phosphate-buffered saline; Tris-saline, 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.5); $NaDodSO_4$ sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; β-APN, β-aminopropionitrile fumarate; MalNEt, *N*-ethylmaleimide; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin (fraction V); DEAE, diethylaminoethyl; CM, carboxymethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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