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Cell-Free Synthesis of Herpes Simplex Virus DNA: Structure of the in Vitro Product and Nucleolytic Degradation†

Bertold Francke

ABSTRACT: The size of viral DNA products synthesized in cell-free DNA synthesis systems from HSV-1 infected BHK cells under different incubation conditions is investigated. In the unfractionated concentrated lysate, preexisting viral DNA maintains its size (20–50 S) during the in vitro reaction and viral DNA synthesized in vitro cosediments with native prelabeled DNA. After denaturation with alkali, the viral DNA sediments between 10 and 40 S with a bias of the in vitro synthesized DNA for shorter size classes. In the absence of additional divalent cations other than Mg^{2+}, the lysate system shows practically no endogenous nucleolytic activity, while in diluted lysates endonucleolytic activity is observed. Endonuclease can be stimulated in concentrated lysates by the addition of Ca^{2+}, while the addition of Mn^{2+} stimulates predominantly an exonucleolytic type of degradation. Isolated nuclei can be rendered free of exonucleases by washing with 60 mM KCl, but they do retain endonuclease activity resulting in a native DNA product of 11 S in size for preexisting and in vitro synthesized DNA. Low ionic strength washes do not remove the exonuclease(s) and result in nuclear systems that rapidly degrade viral DNA. Degradation requires Mg^{2+}, but is not dependent on DNA synthesis. All nucleolytic events in vitro are specific for viral DNA, not affecting cellular DNA. Purified viral and cellular DNA are equally sensitive to degradation by the nuclease present in cytoplasm from infected cells. Cellular DNA contained in isolated chromatin is resistant. After reconstitution with chromatin proteins, both types of DNA become resistant to the nuclease. During preparation of chromatin from infected cells, viral DNA is preferentially removed. The differential degradation of viral and cellular DNA during in vitro synthesis therefore is most likely due to differential protection.

In the preceding communication (Francke, 1977), two systems for cell-free DNA synthesis derived from herpes simplex virus infected BHK cells have been described: an unfractionated lysate and purified nuclei. During the experimental evaluation of the various parameters affecting viral and cellular DNA synthesis in both systems, it became clear that nucleolytic activities can play an important role during the in vitro reaction. The action of nucleases during the in vitro synthesis may have different effects, depending on the type of nucleolytic activity and the extent of degradation: extensive exonuclease action would result in degradation of the in vitro product to acid-soluble material, while an endonuclease alone (by nicking), or in combination with limited exonuclease action (resulting in gaps), might act to stimulate DNA synthesis by creating new primers and exposing new templates. Indications for both have been obtained (Francke, 1977): at late times after infection extensive degradation of preexisting and in vitro labeled DNA was observed, and the addition of cytosol from infected cells to isolated nuclei triggered an excess of DNA synthesis over that observed in the unfractionated lysate. The type and extent of nucleolytic activity predominant under certain incubation conditions is therefore expected to influence greatly the structure of the in vitro product. In this communication, an analysis is presented of the viral DNA product synthesized under conditions that minimize degradation and under a variety of conditions that favor certain types of nucleolytic activities. Whenever observed, the in vitro degradation affected exclusively viral DNA, both preexisting and synthesized in vitro. This apparent specificity is of interest since it might reflect a difference in the intracellular environment of the two types of DNA in infected cells.

Experimental Procedure

Biological procedures, preparation, and incubation conditions for cell-free DNA synthesis systems, CsCl density gradient centrifugation, velocity sedimentation in neutral sucrose gradients, and the sources of chemicals and radioisotopes were as described in the preceding report (Francke, 1977). Velocity sedimentation in alkaline sucrose gradients was as follows: 3.6 mL of linear 5–20% sucrose in 10 mM EDTA,[1] 0.25 M NaOH and 0.75 M NaCl were overlayered with 200 μL of sample, dissolved in 0.25 M NaOH, and centrifuged in a Beckman

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[1] Abbreviations used: HSV-1, herpes simplex virus, type I; pfu, plaque-forming units; Hepes, *N*-2-hydroxyethylpiperazine-*N*′,2′-ethanesulfonic acid; dThd, deoxythymidine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

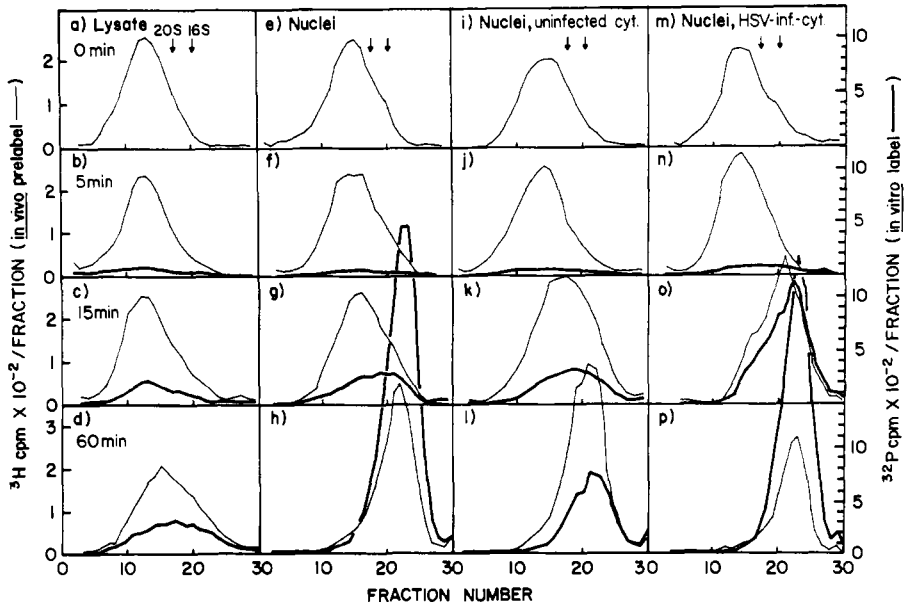

FIGURE 1: Sedimentation profiles in neutral sucrose gradients of HSV DNA after incubation in the lysate and in nuclei, in the absence and presence of cytosol. BHK cells were prelabeled with [^{3}H]dThd (10 μCi/mL) from 11 to 12 h after infection with 10 pfu of HSV/cell at 31 °C. At 12 h after infection, lysate and nuclei were prepared and incubated in vitro as described (Francke, 1977) in the presence of [α-^{32}P]dATP (50 μM, 120 μCi/mL) at 31 °C for the times indicated. Cytosol (Francke, 1977) was from uninfected or HSV-infected (12 h, 31 °C) BHK cells. Viral DNA was separated from cellular DNA by preparative CsCl gradient centrifugation. Fractions containing viral DNA were dialyzed against 10 mM Tris (pH 7.9), 1 mM EDTA, and aliquots sedimented through neutral sucrose gradients in 1 M NaCl as described (Francke, 1977). The zero time samples contained ^{32}P-labeled polyoma DNA forms I (20 S) and II (16 S), as indicated by the arrows. Sedimentation was from right to left. In vivo prelabel (^{3}H, —) and in vitro label (^{32}P, ▬) at 0 (a, e, i, m), 5 (b, f, j, n), 15 (c, g, k, o), and 60 (d, h, l, p) min of in vitro incubation in the lysate (a–d), and nuclei in the absence (e–h) and presence of cytosol from uninfected (i–l) and infected (m–p) cells.

SW56 rotor at 45 000 rpm and 15 °C for 2.5 h. Fractions, 28–30, were collected from the bottom of the tube.

Preparation of Viral Capsids. C-type capsides (derived from virions by treatment with NP-40) were prepared as described by Gibson and Roizman (1972), after labeling infected cells from 4 to 24 h after infection with [^{3}H]dThd (20 μCi per 5 mL per 9-cm dish).

Preparation of Chromatin. Chromatin from uninfected or infected-BHK cells was prepared from nuclei by the procedure of Stein et al. (1976), except that 1 mM Tris-Cl buffer, pH 8.0, was used for the swelling. After the second pelleting, the chromatin was resuspended in 1 mM Tris and adjusted to a concentration of 2 OD_{260} units/mL.

Dissociation and Reconstitution of Chromatin. Chromatin was dissociated in 3 M NaCl, 5 M urea, and 10 mM Tris-Cl, pH 8.3, and the DNA pelleted at 88 000*g* for 24 h (Stein and Farber, 1972). The chromosomal proteins were removed and the tube rinsed gently with 1 mM Tris-Cl. The pellet (containing 97% of the DNA) was dissolved in 1 mM Tris-Cl at 10 OD_{260} units/mL. For reconstitution (Huang and Huang, 1964), the chromosomal proteins, in 3 M NaCl, 5 M urea, and 10 mM Tris-Cl, were mixed with not more than 0.05 volume of DNA in 1 mM Tris-Cl (type and amount of DNA, as detailed in Results). The mixture was dialyzed against decreasing NaCl in 5 M urea and 10 mM Tris-Cl (200 volumes, 3–4 h per step, 4 °C) as follows: 3, 2.5, 2, 1.5, 1, 0.8, 0.6 M NaCl, followed by 0.6 M NaCl in 10 mM Tris-Cl without urea and finally against two changes of 10 mM Tris-Cl. Recovery of input DNA was >75% in all cases.

Nuclease Assay. The nuclease activity of a cytosol preparation from infected cells (prepared as described, Francke, 1977) was assayed in 50 mM Tris-Cl, pH 9.0, 2 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 125 μg/mL of native BHK DNA. Two microliters of concentrated cytosol was used per 100 μL of reaction. Incubation was for 0, 30, 60, 90, and 120 min at 31 °C, at which time 10 volumes of 10 mM EDTA containing 30 μg of calf thymus DNA was added. After addition of an equal volume of 10% trichloroacetic acid, the precipitate was collected onto glass fiber filters, washed twice with 5% trichloroacetic acid, once with 95% ethanol and the radioactivity counted.

Results

The Size of in Vitro Synthesized HSV DNA. When lysates or purified nuclei from HSV-infected cells were incubated under the optimal conditions described in the preceding report (Francke, 1977), viral DNA prelabeled for 1 h in vivo or labeled during the in vitro reaction was found to remain largely stable against nucleolytic degradation to acid soluble material. When examining the size of the native viral DNA at the start and at various times during the in vitro reaction by sedimentation through neutral sucrose gradients, the profiles shown in Figure 1 were obtained. A broad size distribution for the prelabeled DNA was observed, ranging from 20 to 50 S (Figure 1a). It is unlikely that this reflects the actual size of the viral DNA in the cell, since no particular care was taken to avoid shearing during the preparation and collection of the density gradients or during the dialysis (full length HSV DNA from mature virions showed a similar profile, when added after termination of an in vitro reaction and carried through the same procedures). At the start of the reaction, the size of prelabeled DNA was the same in purified nuclei (Figure 1e) as in the lysate, indicating that during the isolation of the nuclei no damage had occurred. During the incubation in the lysate, the prelabeled DNA retained its size and the in vitro label coincided with it, with a slight bias for shorter size classes at late times (Figures 1b–d). In the nuclear system (Figures 1f–h) the short-term in vitro product was indistinguishable from that in the lysate, but during incubation a shift to smaller sizes was apparent. The long-term product in the nuclei sedimented rather uniformly at 11 S. This size class continued to be synthesized in vitro up to 1 h. Prelabeled DNA was also reduced

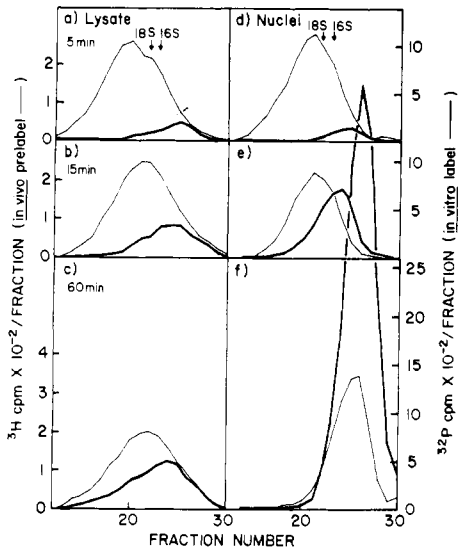

FIGURE 2: Sedimentation in alkaline sucrose gradients of HSV DNA after incubation in the lysate and in purified nuclei. Viral DNA, labeled and prepared as described in legend to Figure 1, was analyzed in alkaline sucrose gradients as described in Experimental Procedure. The top 18 fractions of the 30 fractions collected are shown, the remainder being free of radioactivity. The ^{3}H profiles of the zero-time samples were identical to the 5-min samples and are therefore not shown. ^{32}P-labeled polyoma single-stranded DNA, sedimenting at 18 S (circular DNA) and 16 S (linear DNA), was present in the zero-time sample and its relative position is indicated by the arrows. Sedimentation was from right to left. In vivo prelabel (^{3}H, —) and in vitro label (^{32}P, ▬) at 5 (a, d), 15 (b, e), and 60 (c, f) min of incubation in the lysate (a c) or nuclei (d-f).

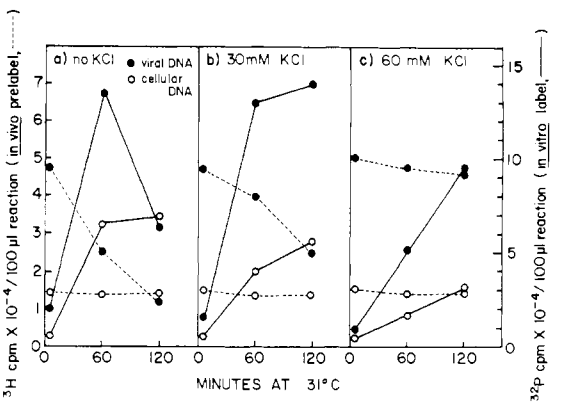

FIGURE 3: Synthesis of viral and cellular DNA in nuclei from HSV-infected BHK cells, washed with different KCl concentrations. Nuclei from cells infected and prelabeled with [^{3}H]dThd as described in legend to Figure 1 were sedimented through 20% Ficoll (Francke, 1977) and washed twice with isotonic buffer containing the indicated amounts of KCl. In vitro labeling with [α-^{32}P]dATP (10 μM, 50 μCi/mL) was in the presence of 80 mM potassium acetate for all three preparations. Viral and cellular DNA were separated by CsCl density gradient centrifugation. In vivo prelabeled (- - -) and in vitro labeled (—) viral (●) and cellular (○) DNA, after washing of the nuclei in the absence (a) or the presence of 30 mM (b) and 60 mM (c) KCl.

to 11 S during the incubation in the nuclear system. When analyzed under denaturing conditions (Figure 2), the initial product in the lysate (Figure 2a) was shorter than the prelabeled DNA and increased during incubation. At late times (Figure 2c) the prelabeled DNA had decreased in size to some extent, and the size distribution of the two labels suggested that single-strand interruptions were present both in the template and the in vitro product. In the nuclear system (Figures 2d-f), after some initial strand growth (Figure 2e) the size after long term incubation (9-10 S) indicated that the predominant product consisted of double-stranded DNA of about 1000 to 1200 base pairs (the in vitro product was completely resistant to the single-stranded nuclease S1 at all times in both systems, data not shown).

In combination with the in vitro synthesis kinetics shown in Figure 13 of the preceding report (Francke, 1977), these observations can be interpreted as follows. In the lysate, in vitro synthesis results in native viral DNA products which sediment similar to the in vitro prelabeled DNA and which show evidence for strand growth during the in vitro reaction, when analyzed under denaturing conditions. Purified nuclei, which continue linear synthesis for longer times than the lysate, produce a short viral DNA product. The fact that in vivo prelabeled DNA also becomes reduced in size during the in vitro reaction in the nuclear system demonstrates the presence of an active endonuclease. Addition of cytosol from uninfected cells (Figures 1i-l), while inhibiting excess in vitro synthesis, did not prevent the endonucleolytic type of degradation in the nuclei. Cytosol from infected cells (Figures 1m-p) accelerated endonucleolytic degradation and caused transient stimulation of synthesis followed by an exonucleolytic type of degradation resulting in loss of acid-precipitable material from prelabeled and in vitro labeled DNA.

When analyzed in a similar fashion, native prelabeled cellular DNA sedimented broadly between 30 S and 80 S and maintained this size distribution throughout the in vitro reaction in both systems under any of the conditions used (not shown). There was no loss of acid-precipitable material from either prelabeled or in vitro labeled cellular DNA.

Nucleolytic Degradation. The degradation of viral DNA appears to be impeded in the lysate, suggesting the presence of an inhibitor of nucleases and/or a protecting agent for the DNA. Such factors appear to be removed during the purification of nuclei along with most of the exonucleolytic activity present in the cytosol of infected cells. This is substantiated by the experiment shown in Figure 3, in which syntheses in nuclei washed with buffers of different ionic strengths were examined. With equal recovery of prelabeled DNA in all three cases, nuclei washed in low ionic strength (Figure 3a) degraded preexisting and in vitro synthesized DNA extensively. Washing with increasing KCl concentrations (Figures 3b, c) resulted in a greater stability of both. When examining the size of the product (gradients not shown), viral DNA at 1 h of incubation was still small (11 S, native) after a 60 mM KCl wash of the nuclei, indicating that endonucleolytic activity washes out less efficiently. The potential protective agent for viral DNA from nucleases, though, appears to diffuse easily at low ionic strength.

The presence of a diffusible factor in unfractionated lysates, protecting viral DNA from endonuclease, is demonstrated by the experiment shown in Figures 4 and 5. If incubated long enough (3 h in Figure 4a), degradation of in vitro synthesized viral DNA was observed even in a concentrated lysate. This was mostly due to an exonucleolytic activity since it was not accompanied by a major change in the size (Figure 5a) and was less prominent in diluted lysates (Figures 4b, c). In the 1:4 diluted lysate though, an endonucleolytic type of activity became apparent (Figure 5b) arguing for a concentration-dependent protecting agent, at least from this type of nucleolytic attack.

The two types of nucleolytic activities respond differently to the presence of divalent cations other than Mg^{2+}. The experiment shown in Figures 6 and 7 was performed in the absence of EGTA, normally included in the reaction mixture. The omission did not affect synthesis in the lysate noticeably when Mg^{2+} was the only divalent cation present (Figure 7a). The

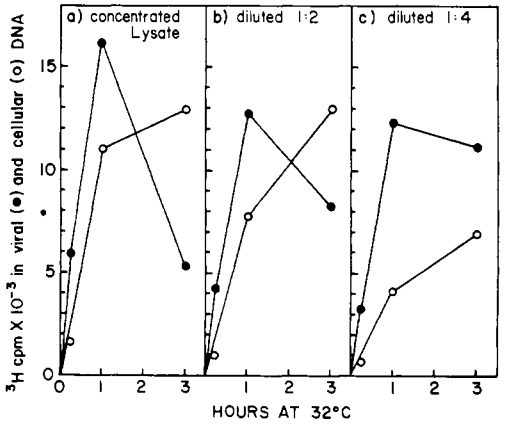

FIGURE 4: Concentration dependence of DNA synthesis in lysates from HSV-infected cells. Lysate (2.5 mL) prepared from infected cells was incubated, concentrated, and 1:2 or 1:4 fold diluted with buffer containing the same concentrations of components as used for DNA synthesis. Labeling was with [^{3}H]TTP (100 μCi/mL, 10 M). Samples removed at the times indicated were analyzed in CsCl density gradients. Viral (●) and cellular (○) DNA labeled in the concentrated lysate (a) and in 1:2 (b) or 1:4 (c) diluted lysates.

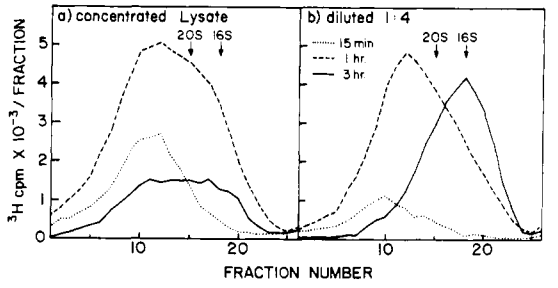

FIGURE 5: Size of the viral DNA synthesized in concentrated and diluted lysates. Experimental details were as described in legend to Figure 4. Neutral sucrose gradient sedimentation was as described in legend to Figure 1. ^{32}P-labeled polyoma DNA was present in all samples and its position was used to align the profiles. 15 min (···), 1 h (- - -), and 3 h (—) viral DNA products in the concentrated (a) and the 1:4 diluted lysate (b).

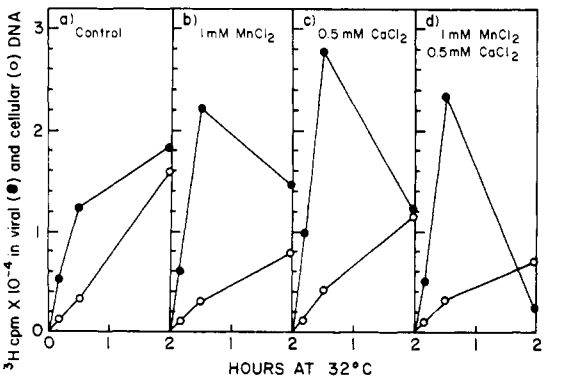

FIGURE 6: Influence of different divalent cations on the synthesis in lysates from HSV-infected cells. Lysate (4 × 0.5 mL) was prepared and incubated under standard conditions except that EGTA was omitted. Total divalent cations were present at 4 mM, $CaCl_2$ and $MnCl_2$ at the concentrations indicated, the remainder being $MgCl_2$. [^{3}H]TTP (100 μCi/mL) was present at 10 μM. Samples (100 μL) were removed at the times indicated and analyzed by preparative CsCl density gradient centrifugation. ^{32}P-labeled polyoma DNA was used as a marker as described in legend to Figure 1. Viral (●) and cellular (○) DNA in the presence of Mg^{2+} only (a), 1 mM Mn^{2+} (b), 0.5 mM Ca^{2+} (c), 1 mM Mn^{2+} and 0.5 mM Ca^{2+} (d).

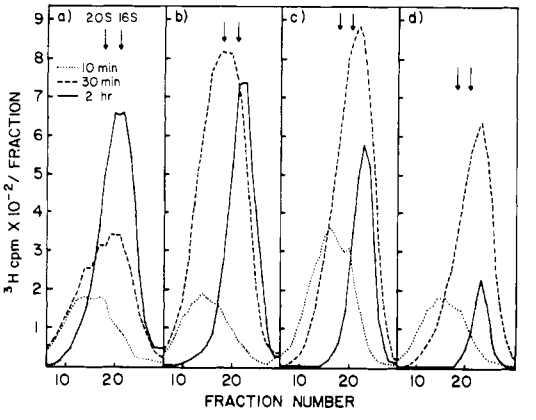

FIGURE 7: Size of the viral DNA product in lysates in the presence of different divalent cations. Experimental details were as described in legend to Figure 6. Viral DNA was analyzed by neutral sucrose gradient sedimentation as described in legend to Figure 5. The top 24 fractions of a total of 28-30 fractions collected are shown. 10 (···), 30 (- - -), and 120 (—) min products, in the presence of Mg^{2+} only (a), Mn^{2+} (b), Ca^{2+} (c), Ca^{2+} and Mn^{2+} (d).

addition of Ca^{2+} (at 0.5 mM) or Mn^{2+} (at 1 mM) resulted in marked degradation of viral DNA after a transient stimulation of synthesis. [The concentrations of Ca^{2+} and Mn^{2+} used were those for optimal synthesis in purified nuclei in the absence of Mg^{2+} (Francke, 1977). Their effect on the synthesis in the lysate shown here was in the presence of Mg^{2+}.] As apparent from the sucrose gradient profiles (Figure 7), the presence of Ca^{2+} (Figure 7c) reduced the size of the product markedly by 30 min, while Mn^{2+} stimulated synthesis without affecting the size of the product. If added together with Ca^{2+}, Mn^{2+} (Figure 7d) accelerated the exonucleolytic degradation to acid-soluble material, probably secondary to the increase in available ends.

Since in many cases nucleolytic activity was accompanied by stimulated DNA synthesis, it was of interest to investigate whether DNA synthesis was required for degradation. The experiment shown in Figure 8 was performed using nuclei washed at low ionic strength (compare Figure 3a), from cells at 15 h after infection—conditions which result in predominant degradation (Figure 8a). When DNA synthesis was prevented by the addition of excess EDTA (Figure 8b), the prelabeled DNA remained stable during incubation, demonstrating a requirement of the nuclease(s) for divalent cations. When DNA synthesis was prevented by the omission of deoxyribonucleoside triphosphates other then TTP (Figure 8c), degradation proceeded at a similar rate and to a similar extent as in the control, documenting that ongoing DNA synthesis was not required for the degradation.

Specificity of Degradation. In all experiments reported so far, nucleolytic degradation—when observed—was specific for viral DNA. This apparent specificity may be due to the ability of the nuclease(s) to differentiate between the two DNA species as such, or to their differential protection in the cell-free systems. To distinguish between the two possibilities the experiment shown in Figure 9 was performed. Cytosol from infected cells was used as a source of nuclease and incubated with various substrates under conditions optimal for the HSV-induced exonucleases (Kier and Gold, 1963). Purified viral and cellular DNA were both fully degradable (Figure 9a). The difference in the rate of degradation was most likely due to a difference in size of the two substrates, which was not controlled in this experiment. Cellular DNA contained in a chromatin preparation was resistant (Figure 9c) but became sensitive when dissociated from the chromatin proteins by high

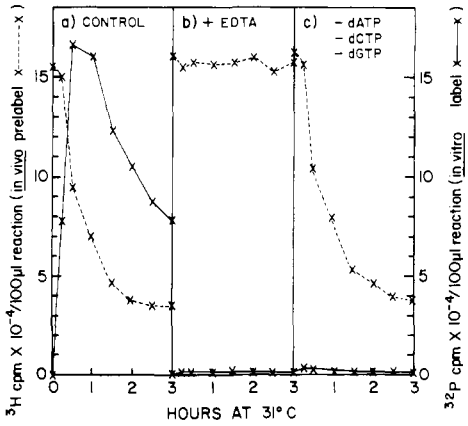

FIGURE 8: Dependence of in vitro degradation on in vitro DNA synthesis in nuclei from BHK cells 15 h after infection with HSV at 31 °C. Nuclei were prepared from cells, prelabeled with [^{3}H]dThd from 14 to 15 h after infection, at 15 h by washing with low ionic strength as in Figure 3a. Three 1-mL reactions were prepared under standard conditions (with [α-^{32}P]-TTP, 25 μCi/mL, 10 μM), in the presence of 10 mM EDTA, and in the absence of deoxyribonucleoside triphosphates other than TTP. Samples were removed at the times indicated and the total radioactivity in acid-precipitable material was determined. ^{3}H cpm (- - - -, in vivo prelabel) and ^{32}P cpm (—, in vitro label) under standard conditions (a), in the presence of excess EDTA (b), and in the absence of dATP, dCTP, and dGTP (c).

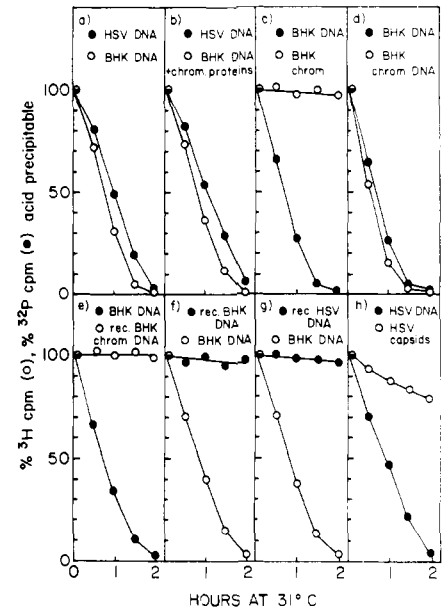

FIGURE 9: Digestion of BHK and HSV DNA, free and associated with chromatin proteins, by extracts from HSV-infected cells. Nuclease assays were carried out as described in Experimental Procedure in the presence of 120 μg of unlabeled BHK/mL. All labeled substrates were present at 0.9 μg/mL. ^{3}H-labeled BHK chromatin DNA (1.54 × 10^5 cpm/μg) was separated from dissociated chromatin as described in Experimental Procedure, after labeling subconfluent BHK cells for 24 h with 50 μCi/mL of [^{3}H]dThd at 37 °C. ^{3}H-labeled (1.87 × 10^5 cpm/μg) or ^{32}P-labeled (3.97 × 10^4 cpm/μg) BHK DNA and ^{32}P-labeled (6.28 × 10^4 cpm/μg)HSV DNA were purified by CsCl density gradient centrifugation, dialyzed, and concentrated by ethanol precipitation. For reconstitution with chromatin proteins, 9 μg of DNA was mixed with 0.3 mL of chromatin proteins in 3 M NaCl and 5 M urea and dialyzed as described in Experimental Procedure (protein:DNA ratio ca. 3:2). Percent ^{32}P cpm (●) and ^{3}H cpm (○) acid precipitable after nuclease digestion for the times indicated: ^{3}H-labeled BHK DNA and ^{32}P-labeled HSV DNA digested in the absence (a) and presence (b) of chromatin proteins, carried through the reconstitution procedure in the absence of DNA; ^{3}H-labeled BHK chromatin (c), the chromatin DNA after dissociation (d) and reconstitution (e), digested in the presence of ^{32}P-labeled BHK DNA; reconstituted ^{32}P-labeled BHK DNA (f) and reconstituted ^{32}P-labeled HSV DNA (g) digested in the presence of ^{3}H-labeled BHK DNA; capsids from [^{3}H]dThd-labeled HSV virions (h) digested in the presence of ^{32}P-labeled HSV DNA.

TABLE I: Distribution of Cellular and Viral DNA during Preparation of Chromatin from HSV-Infected BHK Cells Labeled with [^{3}H]dThd.[a]

^{3}H radioactivity as	Viral DNA	Cellular DNA
Present in nuclei	23.1 × 10^7 cpm (100%)	175.3 × 10^6 cpm (100%)
Removed by washing in 1 mM Tris		
First wash	28.0%	1.3%
Second wash	64.9%	19.9%
Retained in chromatin	6.9%	79.8%

[a] Nuclei were prepared from BHK cells, infected with HSV for 24 h at 31 °C, and labeled with [^{3}H]dThd (25 μCi/mL) from 1 to 24 h after infection as described (Francke, 1977). Nuclei were swollen twice for 30 min at 4 °C in 1 mM Tris-Cl, pH 8.0 (first and second wash). Chromatin is referred to as the pelletable material after the second wash. Aliquots of the nuclei, the washes, and the chromatin were analyzed by CsCl density gradients as described. Results are presented for viral DNA and cellular DNA as percent of the total radioactivity of each present in the nuclei.

salt and urea (Figure 9d). Resistance could be restored by reconstitution with chromatin proteins (Figure 9e). Similarly, purified cellular (Figure 9f) or viral (Figure 9g) DNA was protected from the nuclease after reconstitution with chromatin proteins. For the protection to occur, it was necessary to carry proteins and DNA through the reconstitution procedure (see Experimental Procedure), since mere addition of dialyzed chromatin proteins to the DNA prior to the nuclease assay did not have this effect (Figure 9b). DNA contained in viral capsids was less accessible to the nuclease than free viral DNA (Figure 9h). It is not known which of the proteins released from chromatin by high salt and urea provided the protection or whether the reconstitution scheme used resulted in a chromatin-like structure for cellular and viral DNA. The main points shown by this experiment are that purified cellular and viral DNA show no differential sensitivity to nucleolytic degradation and that DNA contained in a chromatin structure is resistant. The sensitivity of HSV DNA in the cell-free system therefore suggests that the viral DNA in the cell lacks such protection. This is supported by the experiment described in Table I. When chromatin was prepared by standard procedures from infected cells, viral DNA was preferentially lost during the swelling of the nuclei and the washing of the chromatin. The 6.9% of the total viral DNA still present in the "chromatin" after the second wash was preferentially digested when treated with cytosol from infected cells under nucleolytic conditions (Figure 10). Whether the shoulder of radioactivity still found in the gradient at viral density after nuclease treatment of the chromatin was due to resistant viral DNA, single-stranded cellular DNA, or dense cellular satellite DNA was not investigated further.

Discussion

The experiments presented have documented that the structure of HSV DNA synthesized in cell-free systems is not only a consequence of synthetic events, but also subject to various nucleolytic processes. It appears therefore premature to apply criteria for semiconservative replication (Kolber,

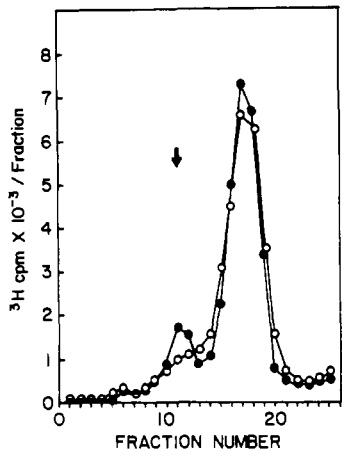

FIGURE 10: CsCl density gradient profiles of DNA from chromatin of HSV-infected cells, before and after digestion of the chromatin with extracts from infected cells. Chromatin from infected BHK cells, labeled with [^{3}H]dThd and prepared as described in the footnote to Table I, was treated with cytosol from infected cells as described in the legend to Figure 9 for 2 h at 31 °C. The DNA was liberated with 2% $NaDodSO_4$ mixed with ^{32}P-labeled HSV marker DNA and analyzed in CsCl density gradients as described (Francke, 1977). Superimposed profiles for the DNA before (●) and after (○) nuclease treatment of the chromatin. The position of the viral DNA marker is indicated by the arrow. Density increase is from right to left.

1975) or RNA-priming (Murray and Biswal, 1974) to systems in which synthesis is complicated by degradation. Another difficulty in ascertaining the validity of a cell-free DNA synthesis system in terms of its relevance for replicative viral DNA synthesis is the current lack of knowledge about the process in vivo. Recent findings by Ben-Porat et al. (1976) and Shlomai et al. (1976) indicate that replication occurs in structures larger in size than mature viral DNA and might proceed bidirectionally from an unique origin. Nothing is known about the mechanism of strand growth. From the size of the in vitro product (Figure 1) the system which is most likely to support synthesis in long concatemers appears to be the lysate, provided it is used concentrated and without the addition of divalent cations other than Mg^{2+}. In this system evidence for strand growth during in vitro synthesis could be obtained (Figure 2). Experiments are in progress to search for high molecular weight DNA structures in the lysate, with the labeling properties of replicating DNA, by minimizing potential shearing during the characterization.

Although undesirable in a cell-free DNA replication system, the nucleolytic activities observed under various conditions in vitro are of interest because of their implications regarding the state of the viral DNA in infected cells. Operationally defined, two types of nucleolytic attack could be differentiated: exonuclease (loss of acid-precipitable material from DNA) and endonuclease (reduction in size of the DNA without loss of acid precipitability). The endonuclease alone was most prominent in the nuclear system (Figure 1) which was almost free of exonuclease if the nuclei had been washed with sufficiently high ionic strength (Figure 3). The size of the native in vitro product (11 S) was very reproducible under these conditions and may be related to the tertiary structure of viral DNA in the nucleus. An endonuclease was detected also in the lysate, when the system was diluted (Figure 5), and could be induced in the concentrated lysate specifically by the addition of Ca^{2+}. The exonuclease was stimulated in the lysate by the addition of Mn^{2+}. This activity was present in the cytosol, specifically from infected cells (Figure 1), but extensive washing was required to remove it from the nuclei (Figure 3).

The conclusion which can be derived from these observations, that two clearly distinguishable nucleases are responsible for all effects, is almost certainly an oversimplification. Theoretically, one enzyme could suffice if it changed its mode of action depending on its concentration and the kind of divalent cations present. On the other hand, many different nucleases may be present, with both endo- and exonucleolytic activities. It is also not known at present whether the activities are of viral or cellular origin. Ca^{2+} stimulated DNA synthesis, secondary to nucleolytic action, has been observed in nuclei from uninfected rat liver cells (Burgoyne et al., 1970), and the corresponding phenomenon observed here (Figure 4) might be caused by a cellular enzyme. Similarly, the stimulation of DNA synthesis in lysates at low pH (Francke, 1977) might be secondary to a nuclease with an optimum at pH 5, present in uninfected BHK cells (Francke, unpublished observations). The majority of the cytoplasmic exonuclease is most likely induced by the virus because of its absence from uninfected cytosol (Figure 1). The known properties of the HSV-induced nuclease (Morrison and Keir, 1968) point to this enzyme as a reason for the stimulation of DNA synthesis in lysates at high pH (Francke, 1977). For HSV-2 a temperature-sensitive mutant exists which lacks alkaline exonuclease activity in extracts from cells infected or shifted to nonpermissive temperature (J. Hay, personal communication). Because this mutant is not temperature-sensitive for viral DNA synthesis, it should be very useful as a tool to investigate the potential role of this viral gene in the various degradation steps during cell-free synthesis.

Since HSV DNA is not known to contain unusual bases, it is unlikely that the differential sensitivity of viral and cellular DNA to nuclease is an inherent property of the two types of DNA. It is more likely that the intranuclear environment of the DNA differs such as to render viral DNA sensitive while protecting cellular DNA. The histones contained in chromatin could serve this purpose for cellular DNA, as borne out by the resistance of DNA in isolated chromatin to the nuclease (Figure 9). The failure to retain HSV DNA in a nuclease-resistant form during a preparation of chromatin from infected cells (Table I, Figure 10) implies that HSV DNA does not exist in a chromatin-like structure in the cell, or that such a structure is less stable if it contains viral DNA. Like cellular DNA, viral DNA does interact with chromatin proteins from uninfected cells resulting in a nuclease-resistant structure (Figure 9).

HSV does not code for histones, and synthesis of histones is turned off in infected cells along with overall cellular protein synthesis. Unless the virus utilizes preexisting histones it must have some other means of protecting its DNA in the cell. Bacteriophage T4, a virus which is in many respects comparable to HSV, codes for a potent exonuclease (Huang and Lehman, 1972) which will degrade single-stranded viral DNA unless protected by the gene 32 protein (Mosig and Bock, 1976). HSV induces a protein with similar DNA-binding properties as the gene 32 of T4 (Bayliss et al., 1975). Both proteins interact specifically with single-stranded DNA, though, while the degradation observed here is of double-stranded DNA. The apparent ease with which the putative protective agent diffuses in a diluted lysate or from nuclei by washing in low ionic strength suggests a low molecular weight substance with little affinity for DNA. HSV virions, unlike papovaviruses, do not contain histones, and it is thought that polyamines present in the virion (Gibson and Roizman, 1971) neutralize the DNA in the viral core. Preliminary experiments using polyamines in cell-free systems for HSV DNA synthesis have resulted in a marked increase in the stability of viral DNA.

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Ovalbumin Gene: Purification of the Coding Strand†

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ABSTRACT: Purified ovalbumin messenger RNA ($mRNA_{ov}$) was employed to isolate the gene coding for ovalbumin from total chick DNA by molecular hybridization. Using $mRNA_{ov}$ covalently linked onto phosphocellulose, the coding strand of the ovalbumin gene was enriched 10 000-fold from sheared chick DNA by affinity hybridization chromatography. This gene sequence was further purified by sulfhydryl-Sepharose column chromatography after hybridization with an excess of mercurated $mRNA_{ov}$. The concentration of the ovalbumin gene sequence in the DNA fraction eluted from the sulfhydryl-Sepharose column by 0.1 M 2-mercaptoethanol was quantitated by saturation hybridization with ^{125}I-labeled $mRNA_{ov}$. When the initial slope of this reaction was compared to that obtained from the reaction between [^{125}I]$mRNA_{ov}$ and full-length complementary DNA ($cDNA_{ov}$) synthesized against $mRNA_{ov}$ using reverse transcriptase, purification of the coding ovalbumin DNA strand from the $mRNA_{ov}$ affinity column purified DNA preparation was 18-fold. The overall purification of the ovalbumin gene from total chick DNA was thus 180 000-fold, and two out of every five DNA molecules in the final preparation contained sequences of the structural ovalbumin gene. There was no apparent degradation of the 5000 nucleotide strands of chick DNA throughout the purification procedure. Since $mRNA_{ov}$ has a complexity of 1890 nucleotides, the resulting DNA was more than twice the length of $mRAN_{ov}$ and should contain DNA sequences tandomly linked to the structural ovalbumin gene, which may play a regulatory role in its expression.

A variety of steroid hormones stimulate growth, differentiation, and other biologic functions of their respective target tissues by exerting their primary action at the transcriptional level (O'Malley and Means, 1974; Woo and O'Malley, 1975; Chan et al., 1973; Harris et al., 1973, 1975, 1976; Sullivan et al., 1973; Palmiter, 1973; Tsai et al., 1975a,b; Schwartz et al., 1975). This conclusion has led workers in the area of hormone action to focus their investigations on various regulatory aspects of specific eucaryotic gene expression. These investigations, however, are hampered seriously by the enormous genomic complexities of eucaryotes. Specific proteins inducible by hormones are generally encoded by unique DNA sequences which may constitute only one-millionth of the entire genome. Consequently, although transcriptional studies using unfractionated chromatin have contributed to our present understanding of eucaryotic gene expression, the exact molecular mechanism of reguation of specific eucaryotic gene expression remains unresolved. In order to perform definitive studies on the regulation on eucaryotic gene expression at the molecular level, the various components of the transcriptional machinery must eventually be purified so that the entire system can be reconstituted in vitro. Such studies require the development of methodology for gene isolation.

Ovalbumin is an egg-white protein synthesized in the chick oviduct in response to estrogen stimulation (O'Malley et al., 1967; Palmiter et al., 1971), and the ovalbumin gene is a unique DNA sequence in the chick genome (Harris et al., 1973; Sullivan et al., 1973). Since this gene possesses identical physical-chemical properties to the bulk of chick DNA (Woo

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