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SYNTHESIS OF HERPES SIMPLEX VIRUS DNA IN A SOLUBLE NUCLEAR EXTRACT

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Summary: A soluble extract prepared from nuclei of HeLa cells infected with herpes simplex virus type 1 has been found to synthesize herpes DNA in a process comparable to that observed in intact nuclei. The incorporation of [^{3}H]dTTP has an absolute requirement for Mg^{++} and for the other three deoxyribonucleoside triphosphates, and is relatively independent of added ATP. The reaction product, although of relatively short chain length, bands in CsCl density gradients at the density of herpes DNA and is essentially free of labeled cell DNA. Incorporation of BrdUTP results in a density shift suggesting extensive replication of endogenous DNA sequences.

Studies of DNA synthesis in cells infected with herpes simplex virus have utilized intact cells (1-7), cell lysates (8,9), and purified nuclei (8-16). Limited information concerning the replication mechanism has been obtained. The inherent insolubility and the restricted permeability of these systems impose limits on the biochemical analysis of the replicative process that might be surmounted in a completely solubilized preparation. Such soluble preparations have recently been reported for SV-40 DNA (17-19) and adenovirus DNA (20-22), and a particulate preparation from disrupted, infected nuclei has been found to synthesize herpes simplex type 1 (HSV-1) DNA (23). While the SV-40 and adenovirus systems primarily synthesize viral DNA, the HSV-1 system appears to incorporate precursors into both host and virus DNA (23). Both HSV and adenovirus are large linear duplexes that replicate in the cell nucleus with the latter virus involving a single-strand displacement mechanism that may be unique to adenovirus (24). This communication deals with the herpes genome and describes a subnuclear, soluble preparation that synthesizes HSV-1 DNA as the predominant product.

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METHODS

Cells and virus: HeLa F cells and the Miyama strain of HSV-1 were obtained from Arthur Weissbach. For the experiments reported here, a clone, HeLa F1 was selected for minimal occurence of giant, multi-nucleated cells and maintained as monolayers in F-15 medium (Grand Island Biological Company) containing 10% heat inactivated fetal calf serum. HSV-1 was prepared from HeLa F1 cells that were infected at 0.1 to 0.2 plaque forming units per cell and maintained in F-11 medium supplemented with 2% heat-inactivated fetal calf serum and 4 mM glutamine until syncytial formation was maximal (48-72 hours). Cells were collected by centrifugation and sonicated after suspension in growth medium at a density of 4 x 10^6 cells/ml. Centrifugation, resuspension of the pellet in a smaller volume, and sonication were repeated several times until most of the original cell pellet had been dispersed. The supernatant solutions were combined on the virus stock and assayed for infectivity by plaque titration of HeLa F1 cells.

Nuclear extract: Cells were harvested 8 hours after infection with 10 plaque forming units per cell, and nuclei were prepared by the method of Berkowitz et al (25). Using a modification of the extraction procedure of Su and Depamphilis (19), nuclei were suspended in hypotonic buffer (10 mM Tris-HCl pH 8.3, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol) at a density of 125 to 150 x 10^6 nuclei/ml and maintained with gentle agitation for 4 hours at 4^o. After centrifugation for 5 min at 8000 rpm, 0.5 to 0.7 ml of nucleus-free solution containing approximately 8 µg of DNA was obtained from 150 x 10^6 cells. Extracts were normally prepared under sterile conditions.

DNA synthesis: Precursor incorporation into DNA was measured at 25^o or 37^o in a total volume of 50 µl containing nuclear extract, 58 mM Tris-HCl pH 8.3, 24 mM KCl, 2.5 mM ATP, 0.2 mM each dATP, dGTP, and dCTP, 5.4 mM $MgCl_2$, 3 mM dithiothreitol, and 60 µM [^{3}H]dTTP containing 10^3 cpm/pmole. Reaction was terminated by immersion in ice and addition of EDTA to 10 mM. For estimates of DNA formation, samples were kept for 1 hour at 60^o in 0.3 M NaOH followed by precipitation with trichloroacetic acid, collection of the precipitate on GF/C glass fiber filters, and scintillation counting of the precipitate.

Density equilibrium sedimentation: Phenol extracted samples in 10 mM Tris-HCl pH 7.4, 100 mM NaCl, and 10 mM EDTA were brought to a refractive index of 1.401 by the addition of solid CsCl. Samples of 8 ml were spun for at least 60 hours at 20^o at 28,500 rpm in the 65 rotor of a Spinco L2-65B centrifuge.

Sedimentation in alkaline sucrose gradients: Gradient solutions containing 5 to 20% sucrose in 4 ml of 0.3 M NaOH, 0.2 M NaCl, and 1 mM EDTA were preformed on top of a 50% sucrose cushion. Samples (200 µl) were layered on top of the gradient and centrifuged in a Spinco SW 50.1 rotor for 3 hours at 45,000 rpm and 4^o.

RESULTS

Synthesis of HSV-1 DNA in soluble extracts has many of the characteristics reported for intact nuclei (8-16). As shown in Fig. 1, the high initial rate of DNA synthesis is dependent upon herpes infection. Incorporation at 37^o continues for 5 min, after which labeled DNA is apparently slowly lost. In experiments with intact nuclei (not shown here) the same kinetic curve is observed except that maximum incorporation occurs at 10 to 15 min rather than

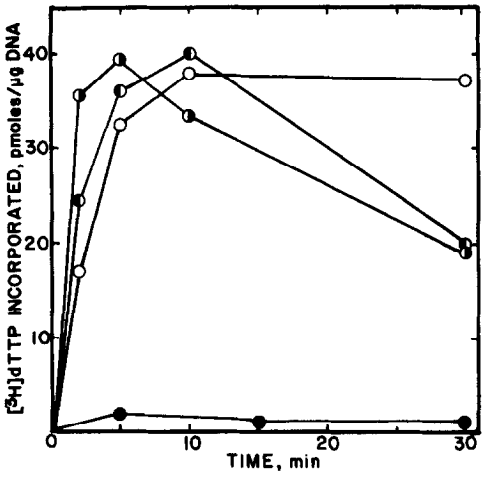


Figure 1. Time course of [^{3}H]dTTP incorporation into DNA. Extracts were prepared from HSV-1 infected HeLa cells and assayed as described in METHODS at several incubation times and temperatures: 37°, ◑; 30°, ◐; and 25°, ○. A corresponding extract from a growing uninfected cell culture was assayed at 37°, ●.

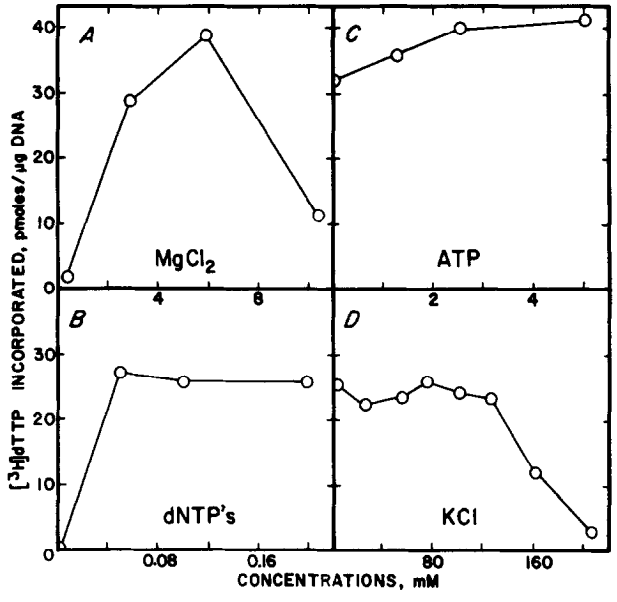
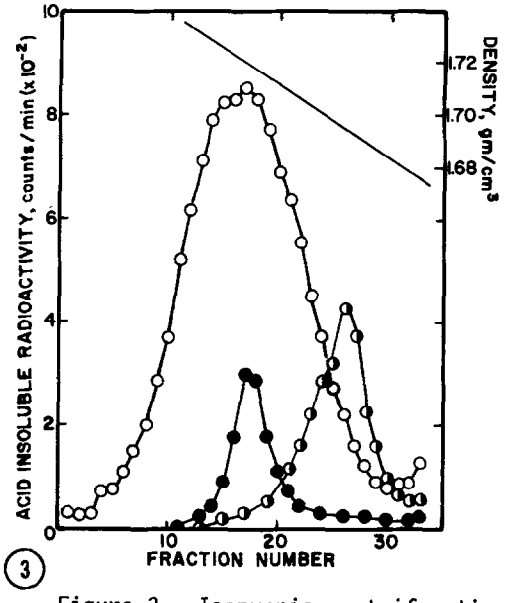


Figure 2. Factors affecting the synthetic rate. Standard incubations were carried out for 5 min at 37° with concentrations of several components varied individually: A, $MgCl_2$; B, dNTP's; C, ATP; and D, KCl.

at 5 min. The apparent degradation of labeled product with time was eliminated by reducing the incubation temperature to 25°.

Some of the requirements for optimal synthesis are illustrated in Fig. 2. There is an absolute dependence upon Mg^{++} (Fig. 2A) and deoxyribonucleoside



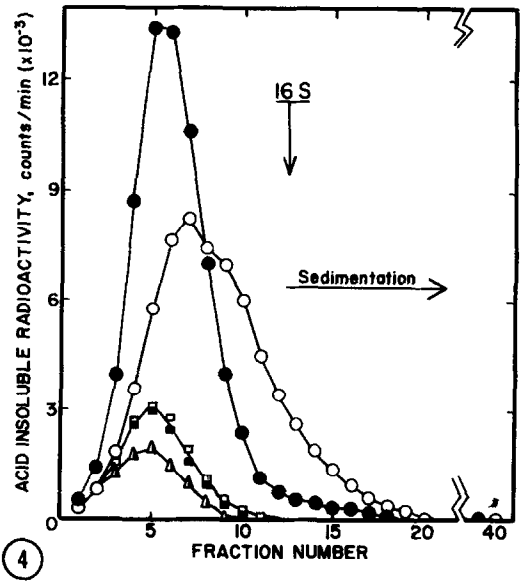


Figure 3. Isopycnic centrifugation in CsCl. A standard incubation with 40 µl of HSV-1 extract was carried out for 5 min at 25° with [^{3}H]dTTP as the labeled substrate. After addition of 50 µg of calf thymus DNA, the mixture was deproteinized with phenol, and aliquots were centrifuged to equilibrium in CsCl (see METHODS) together with authentic [^{14}C]HSV-1 DNA and cell DNA as density markers. Incubation product, ○ ; HSV-1 DNA marker, ● ; and cell DNA, ◑.

Figure 4. Alkaline sucrose gradient sedimentation. Extracts were incubated at 25° for various lengths of time with and without addition of a cytoplasmic extract (100,000 x G supernatant solution prepared from uninfected HeLa cells). Conditions were standard (see METHODS) except that [^{3}H]dTTP was 31 µM with 7,000 counts/min/pmole. Centrifugation conditions are described in METHODS. Closed symbols are with, and open symbols are without cytoplasmic extract. Incubation times were: 0:33 min, △ ; 1.0 min, □ ; and 10 min, ○.

triphosphate (Fig. 2B). ATP is not required although it does stimulate the reaction (Fig. 2C), and KCl has little effect other than inhibition at higher concentrations (Fig. 2D). The preparation is essentially saturated at labeled substrate, [^{3}H]dTTP, concentrations above 10 µM.

The labeled DNA formed in a 5 min incubation was compared with herpes DNA and cell DNA by isopycnic centrifugation in CsCl. While the *in vitro* product bands over a broad density range indicating heterogeneity or low molecular weight, the peak region in Fig. 3 clearly bands with authentic herpes DNA (density, 1.720), and it is distinctly separated from cell DNA.

Attempts to measure chain growth by alkaline sucrose gradient sedimentation of the products of 0.33, 1.0, and 10 min incorporations are shown in

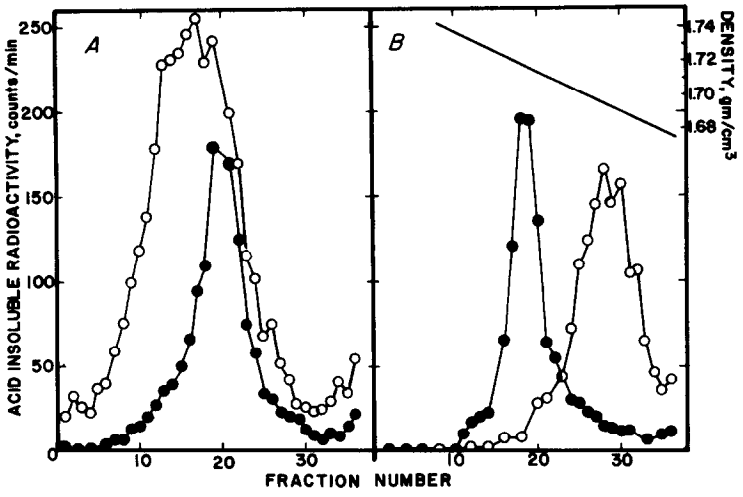


Figure 5. Density shift accompanying BrdUTP incorporation. A. Nuclear extracts from HSV-1 infected cells, labeled with [^{14}C]thymidine from 2 to 8 hours post-infection, were incubated for 10 min at 25° with BrdUTP (61 μM) and [^{3}H]dATP replacing dTTP and dATP, respectively. The product was phenol-extracted and centrifuged in CsCl as described in METHODS. Fractions were acid precipitated and counted: ^{3}H *in vitro* label, ○; ^{14}C pre-label, ●. B. DNA was prepared from the extract prior to *in vitro* incubation and centrifuged as in A: ^{14}C-labeled extract DNA, ●; added ^{3}H-labeled marker cell DNA, ○.

Fig. 4. Small nascent strands (6 to 7S) accumulate with time with no indication of processing to a high molecular weight form. That the preparation may be lacking in factors required for elongation is suggested by the apparent shift to longer heterogeneous strands observed at 10 min when the system was fortified with a cytoplasmic extract from uninfected HeLa cultures.

The extent to which pre-existing strands participated in the synthetic process was estimated by using an extract from infected cells, pre-labeled with [^{14}C]thymidine (Fig. 5B) in an *in vitro* incubation in which BrdUTP and [^{3}H]dATP replaced dTTP and dATP, respectively. The peak to peak density shift of 0.008 g/cm^{3} for newly synthesized DNA shown in Fig. 5A combined with an initial DNA content of 0.60 μg (156 pmoles of dTMP residues) and incorporation of 13 pmoles of [^{3}H]dATP suggests that half of the pre-existing DNA could have been functioning as an active template and that approximately 30% of the final base-pair content in these molecules represented *in vitro* synthesis.

DISCUSSION

A soluble extract prepared from nuclei of HSV-1 infected cells incorporated precursors into HSV-1 DNA. The general requirements of the system, including its relatively small stimulation by added ATP are similar to those reported by others for crude lysates or purified nuclei from HSV infected cells (8, 12, 15, 16). Other characteristics of the soluble preparation that mimic intact nuclei are the disappearance of acid-insoluble labeled product upon prolonged incubation which has been ascribed to a nucleolytic activity associated with HSV infection (9), and the labeling of relatively short nascent strands (6 to 7S, approximately 550 nucleotides) that tend to persist without ligation into longer strands. The indication that a cytoplasmic extract of uninfected cells promoted chain elongation (Fig. 4) suggests that essential components are deficient in the soluble preparation. It is also possible, however, that nucleases of the type observed by Francke (9) may limit chain growth since neutral sucrose gradient sedimentation (not shown) indicated a relatively small size for double stranded labeled DNA (9.6S, approximately 1750 base pairs) that tended to decrease with time.

Buoyant density analysis suggests that the soluble preparation, in contrast to intact nuclei or the chromatin pellet preparation of Yamada et al (23), synthesizes only HSV-1 DNA with no detectable synthesis of host cell DNA. Whether this reflects an extraction selectivity for small DNA fragments or particular replication complexes is not known.

The extensive in vitro incorporation observed in the BrdUTP experiment paralleling the observation of Yamada, et al in total chromatin (23) is indicative of replication rather than repair. This observation combined with high incorporating activity (10 to 20% of that in the nuclei extracted) and low DNA content (0.4 to 0.8% of the nuclear DNA content) suggests the utility of the soluble system in probing the biochemical mechanisms of HSV-1 DNA replication.

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