Replication of herpes simplex virus type I DNA in permeabilized infected cells

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ABSTRACT

Herpes simplex type 1 (HSV)-infected Vero cells can be permeabilized by a combination of hypotonic shock and a mild emulsifier, gum arabic. Permeabilized cells will incorporate triphosphate precursors into viral and host DNA in vitro in ratios similar to those seen in vivo. This reaction is ATP-dependent and is shown to be replicative by the single strand density shift of DNA synthesized in the presence of BrdUTP. The product is heterogeneous in size, and contains a significant proportion of rapidly sedimenting forms and of unit size (55S) viral DNA. The presence of polyamines and EGTA (a specific chelator of Ca^{2+} ions) in the labeling medium is shown to be necessary to maintain the integrity of the replicating DNA. The average size of newly synthesized single strands, however, is smaller than seen in vivo. The reaction is sensitive to phosphonoacetic acid added at the time of labeling, at concentrations which inhibit in vivo synthesis only after one hour of pre-exposure. These properties make permeabilized cell monolayers an attractive system for the study of HSV DNA replication.

INTRODUCTION

A more complete understanding of the mode of replication of eukaryotic and animal virus DNA will depend on the development of subcellular systems that will support processes as similar as possible to in vivo replication. This goal has been achieved to a significant extent for small animal DNA viruses such as polyoma (1,2), SV40 (3,4) and adenovirus (5,6), but remains elusive for larger viruses and whole cells.

A number of infected cell preparations supporting the synthesis of herpes simplex virus (HSV) DNA have been described, including disrupted whole infected cells (7), isolated nuclei (7,8,9,10), nuclear monolayers (11), soluble chromatin (12,13,14), and ill-defined complexes released from infected cell nuclei (15,16). Most of these, however, fail to synthesize unit size viral DNA or to support semi-conservative replication, and none has been shown to incorporate precursors into the large concatemers thought to be the primary templates for HSV DNA replication (17, 18). It has been shown that these

problems were due at least in part to the presence of a Ca^{2+} -stimulated endonucleolytic activity which shows preference for viral DNA (19), and an apparent requirement for polyamines by the viral replicative complex (20).

We reasoned that a more gentle and rapid method for disrupting infected cells might provide a more "physiological" system for the study of HSV DNA replication. For example, the use of a permeable system would obviate the need to account for such factors as selective transport of precursors across the cytoplasmic membrane or size of endogenous precursor pools, and in addition would allow us to conduct pulse-chase or rapid drug addition and removal experiments with great ease. In order to permeabilize infected cells with minimal disruption to the viral replicative apparatus, we chose to use a combination of hypotonic shock and a low concentration of a mild emulsifier, gum arabic. We show here that this method produces a DNA-synthesizing system which is permeable to triphosphate precursors and to phosphonoacetic acid, incorporates label into viral DNA in a replicative fashion, yields both full-size viral DNA and labeled concatemers, and is extremely stable under conditions supporting active DNA synthesis.

MATERIALS AND METHODS

a. Cells and Viruses

Vero and CV-1 cells, originally obtained from Dr. S. Silverstein, Columbia University, were grown as monolayers in minimum essential medium (Earle's base) supplemented with 5% fetal bovine serum, 2mM L-glutamine, and 100 mg/1 neomycin sulfate (MEM 5%). HSV-1, strain MP-CBG, was a kind gift of Dr. B. Roizman. Working stocks were made from plaque-purified virus by low multiplicity (0.01 pfu/cell) infection of CV-1 cells. The virus released into the cell supernatant over 48 hrs was collected by centrifugation and resuspended in a small volume of PBS containing 1% fetal bovine serum and 10% glucose. Stocks used in this study had titers ranging from 4×10^8 to 5×10^9 pfu/ml.

b. Preparation of permeabilized infected cells and labeling of DNA

Confluent monolayers of Vero cells were infected with 5-10 pfu/cell of HSV-1 in MEM 2%. At 6-7 hrs after infection, the cells were treated as follows: the culture fluid was aspirated and the dish put on ice. The cells were rinsed once with ice-cold hypotonic buffer: 50mM HEPES-KOH pH 7.6, 6mM KCl, 5mM MgCl_2 , 2mM ethyleneglycol-bis-(8 aminoethyl ether) N,N'-tetraacetic acid (EGTA), 0.5mM spermine, 0.3mM spermidine, 1mM dithiothreitol, and then

exposed to hypotonic buffer containing 0.5% gum arabic (Sigma) for 3-5 minutes at 0°C. After 2 rinses with cold hypotonic buffer, the cell monolayer was overlaid with labeling buffer: hypotonic buffer containing 0.1mM each dATP, dGTP and dTTP, 2mM ATP and 10-20 µCi/ml of [α -³²P]dCTP (>300 Ci/mmol, New England Nuclear, Boston), and incubated at 37°C. The reaction was stopped by aspirating the labeling buffer and adding ice-cold TE buffer (10mM Tris-HCl pH 7.4, 10mM EDTA).

c. Velocity sedimentation analysis

Cells were scraped into a small volume of ice-cold TE buffer and NaCl was added to a final concentration of 1M. The cells were disrupted by the addition of N-lauroyl sarcosine to a concentration of 1% and 2-3 min. of incubation at 65°C. Pre-digested Pronase (Sigma) was added to a concentration of 1 mg/ml, and the mixture incubated for 4 hrs at 37°C. A 200 µl aliquot of the deproteinized lysate was layered, very slowly and using a wide-mouthed pipette tip, on top of a 10-30% (w/v) sucrose gradient in 1M NaCl, 50mM Tris-HCl pH 7.4, 1mM EDTA, 1% N-lauroyl sarcosine, 0.5% Brij 58 (neutral gradient) or 0.7M NaCl, 0.3M NaOH, 1mM EDTA (alkaline gradient). The neutral gradients were layered over a 0.5ml cushion of 50% Conray (Mallinckrodt). The gradients were spun for 3 hrs (neutral) or 6 hrs (alkaline) at 40000 rpm in a Beckman SW40Ti rotor at 20°C. Fractions were collected from the bottom of the tube and 50 µl aliquots processed for determination of TCA precipitable radioactivity.

d. Isopycnic equilibrium sedimentation

Cells were harvested, lysed and Pronase digested as described above, except that only 0.1 M NaCl was added. The DNA was extracted once with buffer-saturated phenol and once with 2% isoamyl alcohol in CHCl₃, the volume adjusted to 3ml, and solid CsCl added to a density of 1.71-1.72. For alkaline gradients, NaOH was added to a concentration of 0.3M, and the density adjusted to 1.80. The samples were spun for 60-70 hrs at 35000 rpm in a Beckman type 65 rotor at 20°C and fractionated as before.

RESULTS

a. Conditions for DNA synthesis

Our labeling medium was adapted from those described by Tsai & Green (21) and by Bell (11) for NP40-treated nuclear monolayers. In order to maintain the permeability of the treated cells, we considerably lowered the total os-

molarity and ionic strength of the buffer. As can be seen in Table 1 (lines 2 and 3), the addition of ammonium sulfate to the labeling buffer drastically reduces the ability of the cells to incorporate [α - 32 P]dCTP, even though similar concentrations are known to activate the HSV-specific DNA polymerase in other systems (13, and our unpublished observations). Since we can observe a distinct shriveling of the HSV-induced cell-syncytia after increasing the salt concentration or the osmolarity (by adding sucrose), we conclude that the hypotonicity of the labeling medium is essential to maintain a high permeability.

Table 1, line 4, shows that the level of incorporation is relatively independent of added deoxyribonucleotide triphosphates. We interpret this to mean that the endogenous pools of triphosphates are not entirely washed out under our conditions. Line 5, on the other hand, shows that DNA synthesis is ATP dependent, and that the residual pool of ATP is insufficient to support continued synthesis at maximal levels. This conclusion is strengthened by our observation that at shorter incubation times, this dependence on exogenous ATP is not seen. It is generally believed that dependence on ATP as an energy source is a characteristic of replicative synthesis (22).

We added polyamines to our system on the strength of Francke's observation that HSV-specific DNA replication is enhanced in the presence of 0.5mM spermine and 0.3mM spermidine, while repair synthesis is drastically reduced,

TABLE 1
Effects of various factors on DNA synthesis
in permeabilized HSV-infected cells^a

Labeling conditions	Relative incorporation
Control ^b	100%
+50mM(NH ₄) ₂ SO ₄	55%
+100mM(NH ₄) ₂ SO ₄	9%
-dNTP	83%
-ATP	56%
-polyamines	152%
-polyamines, ATP	83%
-EGTA	91%
+5mM EDTA	6%

^a HSV-1 infected Vero cells were disrupted by hypotonic shock at 6 hrs p.i. and incubated for 30 minutes with labeling buffer containing 10 μ Ci/ml [α - 32 P]dCTP, modified as shown. The cells were lysed with sarkosyl and TCA precipitable counts were determined.

^b control: labeling buffer was used as described in Materials and Methods

resulting in an overall reduction in the rate of DNA synthesis (20). Our results (Table 1, lines 6 and 7; see above) show a similar trend, although ATP dependence does not seem to be lost in the absence of polyamines as is the case in isolated nuclei (20). EGTA, a Ca^{2+} -specific chelator, was added in an attempt to minimize degradation of the DNA by the endonuclease described by Francke (19). As can be seen from line 8, EGTA does not have a significant effect on incorporation levels. EDTA, however, almost totally inhibits DNA synthesis (line 9).

Cell monolayers permeabilized and incubated under our standard conditions (see Materials and Methods) are stable for at least 90 min. at 37°C and probably longer. The cells remain attached to the growth surface firmly enough to allow changes of buffer throughout this period. Fig. 1 shows that incorporation proceeds linearly for at least 40 min, which puts all the experiments performed in this study within the range of linear incorporation. It also shows that there is no detectable incorporation into mock-infected cells, presumably because they are resistant to the conditions used for preparing permeable infected cells.

b. Identity of the product

In order to determine the relative incorporation of label into viral and host DNA, we analyzed the DNA synthesized in permeabilized cells in neutral CsCl gradients. The difference in buoyant density between host (1.700–1.705) and viral (1.725) DNA allows their clear separation and independent quantitation. A culture of infected cells was labeled in vivo with $[^3\text{H}]\text{TdR}$ starting at 4.5 hr post infection, a time when host DNA synthesis is still quite active. At 6 hr p.i., cells in the culture were disrupted and labeled in vitro with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ for 30'. At this time (6–6.5 hr p.i.) host DNA syn-

Fig. 1 Time course of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ incorporation

Dishes of HSV-1 infected or mock-infected Vero cells were disrupted by hypotonic shock at 7.5 hours after infection and exposed to $10\mu\text{Ci/ml}$ of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ in labeling buffer at 37°C . After various incubation times, the cells were lysed, the DNA sheared, and TCA-precipitable counts determined. Open circles: mock-infected cells. Closed circles: HSV-infected cells.

thesis has been drastically inhibited as judged by *in vivo* labeling experiments (data not shown). Fig. 2, panel A, shows the result of this experiment. The density profile of *in vivo* ^3H -labeled DNA reflects significant incorporation into host DNA, with a peak of viral DNA clearly evident. In contrast, the density profile of *in vitro* ^{32}P incorporation demonstrates that most of the label is in DNA of viral density. When experiments in which cultures were sequentially labeled *in vivo* and *in vitro* were performed at later times, results demonstrated virtually superimposable profiles, with the great majority of both labels in viral DNA. Thus the monolayer system is capable of faithfully reproducing the *in vivo* situation with respect to the proportions of label entering host or viral DNA. The fact that most of the label is incorporated into viral DNA at the time chosen for most of our experiments (6-7 hrs p.i.) rules out the possibility of "random" repair synthesis, since host DNA makes up most of the mass of the DNA.

c. Identification of viral DNA synthesis as replication

Fig. 2, panel B, shows the density profile in neutral CsCl of DNA synthesized in the presence of 5mM BrdUTP. While no dramatic shift in the den-

Fig. 2 Density profiles of prelabeled DNA and DNA synthesized *in vitro*

Vero cells infected with HSV-1 were labeled from 4.5 to 6 hrs after infection with 20 $\mu\text{Ci/ml}$ of $[\text{H}]$ -thymidine. At 6 hrs p.i., the cells were disrupted and incubated for 5 min. at 37°C in hypotonic buffer without triphosphates or ATP, and then for 30 min. in labeling buffer containing 40 $\mu\text{Ci/ml}$ of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$. Panel A: standard labeling buffer. Panel B: labeling buffer containing 5mM BrdUTP instead of dTTP. The DNA was then extracted and analyzed on neutral CsCl gradients. The horizontal bars indicate the fractions pooled for analysis on alkaline CsCl gradients.

sity of the product is observed, it can be seen that the peak of viral DNA is appreciably skewed toward heavier densities while that of host DNA is essentially unchanged. In order to further analyze these shifts, we pooled the peak fractions of viral DNA as shown by the horizontal bars and analyzed them on alkaline CsCl gradients. The rationale for this was that the density difference between continuously synthesized (replicated) and repaired strands would be enhanced after denaturation. Newly replicated strands should be extensively substituted with BrdU, while repaired strands should show no or almost no shift in their density. The result of this experiment (data not shown) was that viral DNA synthesized in vitro in the presence of BrdUTP sedimented at a substantially higher density than its prelabeled counterpart, while the profile obtained for DNA labeled in the presence of dTTP was virtually superimposable to that of in vivo labeled DNA. Also, the prelabeled DNA showed no significant shift towards higher densities when BrdUTP was included in the labeling medium. Taken together, these results strongly suggest that most of the in vitro synthesis is replicative.

d. Size distribution of replicated viral DNA on neutral sucrose gradients

The size distribution of DNA from infected cells sequentially labeled in vivo and in vitro was analyzed on neutral sucrose gradients under conditions in which full-size viral DNA (55S) sediments a little farther than half-way down the centrifuge tube. Fig. 3, panel A, shows that the DNA can be subdivided into 3 size classes: rapidly sedimenting material that pellets onto the Conray cushion, a peak at the size of viral DNA, and smaller, heterogeneously sedimenting forms. The peak of prelabeled DNA sedimenting slower than 55S probably represents the mean size of randomly degraded DNA. Peak fractions from each of these regions were pooled (horizontal bars) and analyzed on neutral CsCl to determine their relative content of viral and host DNA. Table 2 shows the results of this experiment. It can be seen that both the rapidly sedimenting and small regions of in vitro labeled DNA are enriched for viral DNA (63% and 78% of the counts, respectively) relative to the peak centered at 55S (45% of the counts). This is not too surprising if one considers that after a 30 min in vivo pulse, almost all the label will be found in rapidly sedimenting rather than mature, unit size DNA (Table 3, column 4) and that the sedimentation profiles we observe are the result of overlapping patterns of synthesis and degradation. The important fact is that an appreciable amount of label is incorporated into rapidly sedimenting viral DNA. While some in vitro labeled DNA might be trapped with high molecular weight

Fig. 3 Sedimentation profiles of prelabeled and in vitro labeled DNA on neutral and alkaline sucrose gradients

HSV-infected Vero cells were labeled from 6.5 to 7 hrs after infection with $100\mu\text{Ci/ml}$ of $[^3\text{H}]\text{TdR}$. The cells were then disrupted and the newly synthesized DNA was labeled for 30 min. with $20\mu\text{Ci/ml}$ of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$. The DNA was purified by Pronase/Sarkosyl digestion and analyzed on neutral (panel A) or alkaline (panel B) sucrose gradients. The horizontal bars indicate the fractions pooled for further analysis on neutral CsCl gradients (results shown in Table 2).

TABLE 2

Relative amounts of labeled DNA of viral and host origin in various size fractions^a

	^3H cpm (pre label)		^{32}P cpm (in vitro label)	
	viral	host	viral	host
I ^b	77%	23%	63%	37%
II	52%	48%	45%	55%
III	ND ^c	ND	78%	22%

^aSize fractions from the sucrose gradients shown in Fig. 3 were pooled and analyzed by equilibrium sedimentation in CsCl . The relative amounts of host and viral DNA in the sample were determined by measuring the areas under the peaks centered at $\rho=1.725$ (viral) and $\rho=1.705$ (host).

^bthe Roman numerals refer to the pools indicated by horizontal bars in Fig. 3

^cND: not determined

host DNA, we are inclined to believe that this cannot account for all of the radioactivity found in the pellet. First, marker T_4 DNA mixed with the cells before lysis and extracted together with the total DNA sedimented as a sharp peak at 60S and showed no detectable trapping (data not shown). Second, the total amount of DNA loaded (5 μ g) was well below the maximum capacity of the sucrose gradients.

e. Effects of polyamines and EGTA on the size distribution

In order to determine whether EGTA and polyamines actually do inhibit degradation of labeled DNA, we analyzed the sedimentation profiles of DNA labeled in the absence of one or the other. Table 3 shows how this affected the relative number of counts found in the three regions of the gradient described above. It can be seen that omission of one or the other factor from the labeling buffer dramatically shifted the distribution of in vitro labeled DNA toward smaller sizes. The number of counts in the pellet was reduced from 31% to 6% or 17%, while the proportion of counts sedimenting slower than the 55S peak went up from 43% to 64% or 62%. Prelabeled DNA also suffered some degradation, albeit to an average size somewhat larger than the in vitro labeled DNA. Examination of the total counts in prelabeled DNA before (no incubation) and after in vitro incubation shows that under our standard conditions some residual [3 H]-labeled dTTP may still get incorporated. In the

TABLE 3

Effects of polyamines and EGTA on the size distribution of prelabeled and in vitro synthesized DNA^a

		Labeling conditions			
		standard	-polyamines	-EGTA	no incubation ^b
prelabel (3 H cpm)	pellet	81%	58%	84%	90%
	55S peak	7%	16%	6%	5%
	<55S	8%	18%	12%	1%
	total cpm	31,069	19,245	19,650	20,858
<u>in vitro</u> label (32 P cpm)	pellet	31%	6%	17%	-
	55S peak	14%	18%	11%	-
	<55S	43%	64%	62%	-
	total cpm	63,324	57,505	43,094	-

^a The DNA was labeled and analyzed in neutral sucrose gradients as described in Materials and Methods and the legend to Fig. 3. The proportions of total TCA precipitable counts found in different regions of the gradients were calculated.

^b The DNA was extracted from permeabilized cells immediately after treatment.

absence of EGTA or polyamines, however, either no such incorporation occurs, or, more likely, some proportion of the prelabeled DNA gets degraded to non-TCA-precipitable material.

The effect of EGTA on the size distribution leads us to believe that even our mild permeabilization conditions are sufficient to activate the Ca^{2+} -stimulated endonuclease described by others (19). The effect of polyamines is more difficult to ascribe to a single mechanism. One could tentatively implicate a combination of stabilization of the viral replicase complex and/or binding of polyamines to DNA partially stripped of its normal associated proteins. The important point to note is that in the presence of both EGTA and polyamines, the prelabeled DNA suffers relatively little degradation (Table 3, compare columns 1 and 4).

f. Analysis of the product on alkaline sucrose gradients

The sedimentation profiles of prelabeled and in vitro labeled DNA analyzed in alkaline sucrose are presented in Fig. 3, panel B. It can be seen that the single-strand size of the in vitro labeled product is markedly smaller than that of the prelabeled material. This seems to be a characteristic of disrupted HSV-infected cells, since all in vitro systems synthesizing HSV DNA described so far show this same discrepancy. Clearly, even relatively minor damage to the cell's membranes destabilizes the replicative complex enough to shorten the average stretches of continuous synthesis. An additional factor is that the average single-strand size we observe for DNA pulsed in vivo for 30 min is somewhat smaller than what is seen for the same DNA after in vitro incubation (data not shown).

We consistently observe a lengthening of the prelabeled single strands after in vitro incubation, which would suggest that some ligating activity is still present in the disrupted cells. Surprisingly, the largest average size is seen after incubation in the absence of EGTA, suggesting that this ligating activity might be partially Ca^{2+} -dependent.

g. Effects of phosphonoacetic acid on DNA synthesis

In order to test the permeability of the treated HSV-infected cells to drugs, we investigated the effects of phosphonoacetic acid (PAA) on the level and specificity of DNA synthesis in our system. PAA has been reported to be a specific inhibitor of the HSV-induced DNA polymerase (23). Table 4 shows that DNA synthesis in our system is indeed sensitive to the action of PAA, and the concentration needed to achieve a 50% inhibition of DNA synthesis

TABLE 4

Effects of phosphonoacetic acid on DNA synthesis in permeabilized cells^a

PAA concentration	relative incorporation	% viral DNA
0	100%	64%
10 ⁻⁶ M	97%	ND
10 ⁻⁵ M	64%	ND
10 ⁻⁴ M	20%	53%
10 ⁻³ M	5%	ND
10 ⁻² M	<1%	ND

^a HSV-infected cells were permeabilized at 6 hrs p.i. and incubated for 20' with labeling buffer containing 10 μ Ci/ml of [α -³²P]dCTP and varying concentrations of PAA. The amount of TCA-precipitable ³²P counts was determined. The DNA samples synthesized without PAA or in the presence of 10⁻⁴M PAA were phenol extracted and analyzed in neutral CsCl gradients to determine their content in labeled viral DNA.

(~20 μ M or ~3 μ g/ml) is intermediate between those reported for in vivo activity (24) and activity on a partially purified HSV-specific DNA polymerase (23). Surprisingly, the relative amount of viral DNA synthesized in the absence of PAA (64%) is almost the same as that synthesized in the presence of a concentration of PAA (10⁻⁴M) sufficient to cause an 80% reduction in overall DNA synthesis (53%). In order to ascertain whether this was an artifact introduced by our method of disrupting cells, we performed the same experiment in intact cells. While a one-hour pre-incubation in PAA-containing medium was necessary to allow the drug to penetrate the cells, the effect on overall DNA synthesis was virtually identical, i.e. host DNA synthesis was inhibited to the same extent as viral DNA synthesis. In light of the prevailing views about the specificity of PAA (10, 13, 23), we think that this result warrants further investigation. Of special interest is the observation that isolated polymerase α , which is thought to be the enzyme primarily responsible for host DNA replication, is as sensitive to PAA as the viral enzyme (10). Within the scope of this report, however, the important fact is that the hypotonically shocked cells are permeable to PAA, and that its effects are indistinguishable from those induced in intact cells.

DISCUSSION

HSV-1 infected cells permeabilized by hypotonic shock continue to synthesize DNA in a manner very similar to their intact counterparts. The treatment renders them permeable to triphosphate precursors and to at least

one drug, PAA. DNA synthesis requires divalent cations (but not Ca^{2+}) and ATP. The proportion of viral and host DNA synthesized parallels that seen in intact cells at similar times after infection. Most of the viral DNA synthesis seems to be replicative in nature. The product, while smaller on the average than seen in intact cells, contains a significant amount of rapidly sedimenting forms. The effects of PAA on DNA synthesis parallel those seen in vivo under similar conditions.

Our primary goal in this study was to develop a system mimicking in vivo HSV DNA synthesis as closely as possible while being permeable to a range of exogenous substances. Preliminary in vivo experiments have shown that pulse-labeled replicating HSV DNA molecules are "endless" (our unpublished observations), thus strongly implicating the rapidly sedimenting concatemers as the primary templates for viral DNA replication. The system described in this report is the first in vitro system which incorporates labeled precursors into rapidly sedimenting ($>200\text{S}$) forms. In addition to this obvious difference between this method and previously reported methods, our permeabilized cells are very easy to prepare, and show excellent stability even when prepared at relatively late times after infection, when cell nuclei are notoriously fragile. We believe that these features make our system attractive to use for any study of HSV DNA replication requiring rapid changes in the composition of the environment (such as pulse-chase or drug removal experiments) or access to the replicative complex by compounds that would not normally enter the cell.

We are currently using permeabilized cells to study the first rounds of DNA replication occurring after removal of PAA, in an attempt to gain some understanding of the processes leading from unit length parental DNA to concatemers. Attempts to do this in vivo using $[^3\text{H}]\text{TdR}$ as a label (our unpublished observation) have been unsuccessful so far, presumably because the intracellular pool of the drug has a long clearing time, or because the very large pool of dTTP(25) prevents efficient labeling of the few molecules initiating replication. These problems are easily overcome in disrupted cells.

In summary, cells permeabilized by hypotonic shock offer a convenient and powerful experimental system for the study of some aspects of HSV DNA replication and possibly transcription. More extensively fractionated preparations, however, will be needed to study the structure and enzymology of the replicative complex.

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