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Relationship between Vaccinia Virus Intracellular Cores, Early mRNAs, and DNA Replication Sites

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Virus assembly, a late event in the life cycle of vaccinia virus (VV), is preceded by a number of steps that all occur in the cytoplasm of the infected host cell: virion entry, delivery of the viral core into the cytoplasm, and transcription from these cores of early mRNAs, followed by the process of DNA replication. In the present study the quantitative and structural relationships between these distinct steps of VV morphogenesis were investigated. We show that viral RNA and DNA synthesis increases linearly with increasing amounts of incoming cores. Moreover, at multiplicities of infection that result in 10 to 40 cores per cell, an approximately 1:1 ratio between cores and sites of DNA replication exists, suggesting that each core is infectious. We have shown previously that VV early mRNAs collect in distinct granular structures that recruit components of the host cell translation machinery. Strikingly, these structures appeared to form some distance away from intracellular cores (M. Mallardo, S. Schleich, and J. Krijnse Locker, Mol. Biol. Cell 12:3875-3891, 2001). In the present study the intracellular locations of the sites of early mRNA accumulation and those of the subsequent process of DNA replication were compared. We show that these are distinct structures that have different intracellular locations. Finally, we study the fate of the parental DNA after core uncoating. By electron microscopy, cores were found close to membranes of the endoplasmic reticulum (ER) and the parental DNA, once it had left the core, appeared to associate preferentially with the cytosolic side of those membranes. Since we have previously shown that the process of DNA replication occurs in an ER-enclosed cytosolic "subcompartment" (N. Tolonen, L. Doglio, S. Schleich, and J. Krijnse Locker, Mol. Biol. Cell 12:2031-2046, 2001), the present data suggest that the parental DNA is released into the cytosol and associates with the same membranes where DNA replication is subsequently initiated. The combined data are discussed with respect to the cytosolic organization of VV morphogenesis.

Vaccinia virus (VV) is the best-studied member of the *Poxviridae*, a family of double-stranded DNA viruses that replicate in the cytoplasm of the host cell (21). The cytoplasmic life cycle of VV is complex and consists of distinct stages that can be roughly divided into virion entry, early transcription, and DNA replication, followed by virus assembly and release. Although all these steps occur in the cytoplasm of the infected host cell, relatively little is known how these different processes are organized or how they are structurally related. The aim of the present study is to address some of these questions. They relate to a number of recent observations that we have made with respect to the cytoplasmic organization of VV early mRNAs and of viral DNA replication (19, 29).

Although the mechanism of VV entry is poorly understood and controversial, it is generally accepted that the final result of this process is the delivery of VV cores, lacking viral membranes but containing the viral genome and associated enzymes, into the cytoplasm (7, 18, 24, 27, 31, 32). Shortly after their delivery, these cores start making a defined set of early mRNAs in which about half of the genome is transcribed. In vitro reconstitution of this process has demonstrated that early mRNAs are first made inside the core, from which they are subsequently extruded in an ATP-dependent manner (14). The generally accepted idea is that in infected cells viral early mRNAs are also made inside the core, from which they are extruded to associate with polyribosomes (11, 20; also see below). It is assumed that during this process the genome remains inside the core and that protein synthesis, early in infection, is required for release of the parental DNA from the core (10, 13, 15, 26). This assumption is, however, based on indirect evidence, and it has not been directly demonstrated that the genome remains inside the core during the process of early transcription in infected cells (as is explained in more detail below).

In a recent study we have monitored the intracellular fate of of VV early transcripts. They were visualized by transfecting infected cells either with BrUTP or with a biotinylated antisense oligonucleotide corresponding to the H5R mRNA, followed by confocal microscopy. VV early mRNAs were shown to accumulate in large granular structures that recruited polyribosomes and other components of the host cell translation machinery, implying that they were active in protein synthesis (19). By electron microscopy (EM), the sites where early VV mRNAs accumulated appeared as rather amorphous structures surrounded by polyribosomes but not obviously attached to membranes. Two striking observations were made during that study. First, over the time of infection, the structures appeared to grow in size; second, the mRNA structures accu-

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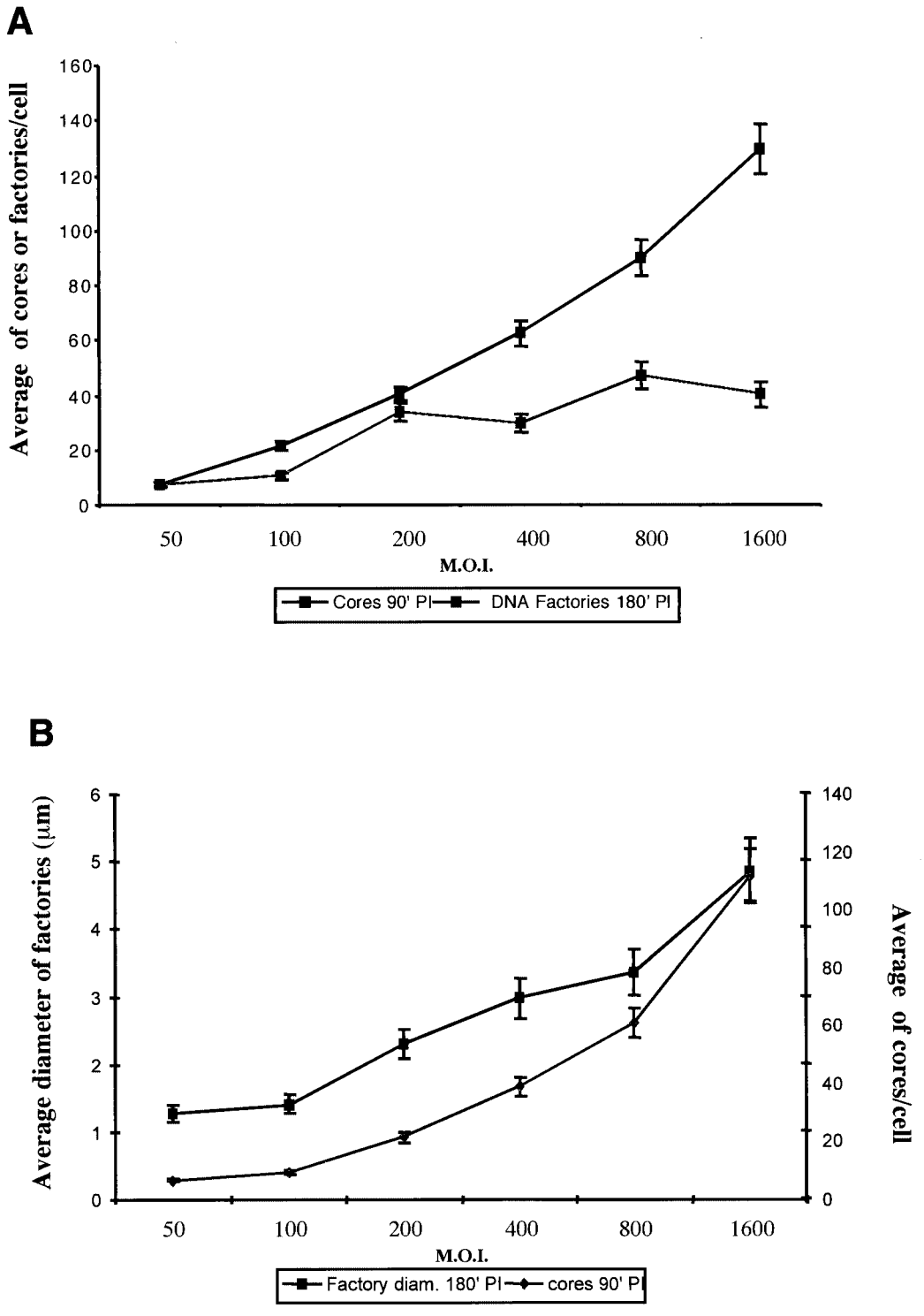


FIG. 1. Relation between MOI, the numbers of intracellular cores and sites of DNA replication, and the sizes of DNA replication sites. (A) Cells grown on coverslips were infected for 15 min at 37°C at the indicated MOI and fixed at 90 min postinfection (PI) in the presence of cycloheximide and 180 min postinfection without cycloheximide. Intracellular cores were counted at 90 min postinfection after labeling with the anti-core antibody, while replication sites were counted at 180 min after labeling with anti-p35. Values are average numbers ± standard errors of the means of cores or replication sites per cell in 30 cells and from three independent experiments. (B) Cells were infected at increasing MOIs, and intracellular cores counted in the same way as for panel A. The diameters of all the factories in 10 cells were measured with NIH Image at 180 min postinfection. Values are average diameters (in micrometers) ± standard errors of the means of all the factories in 10 cells.

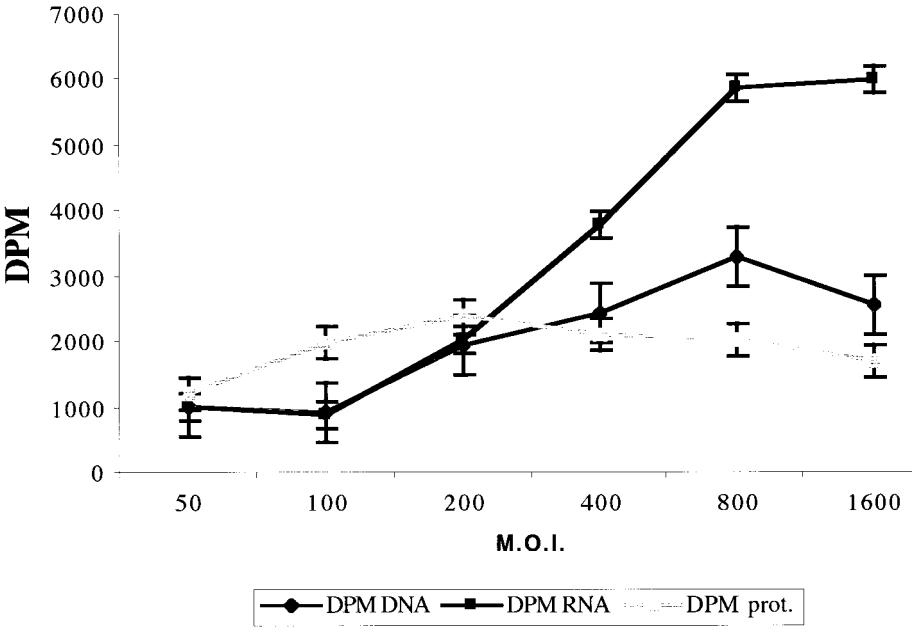


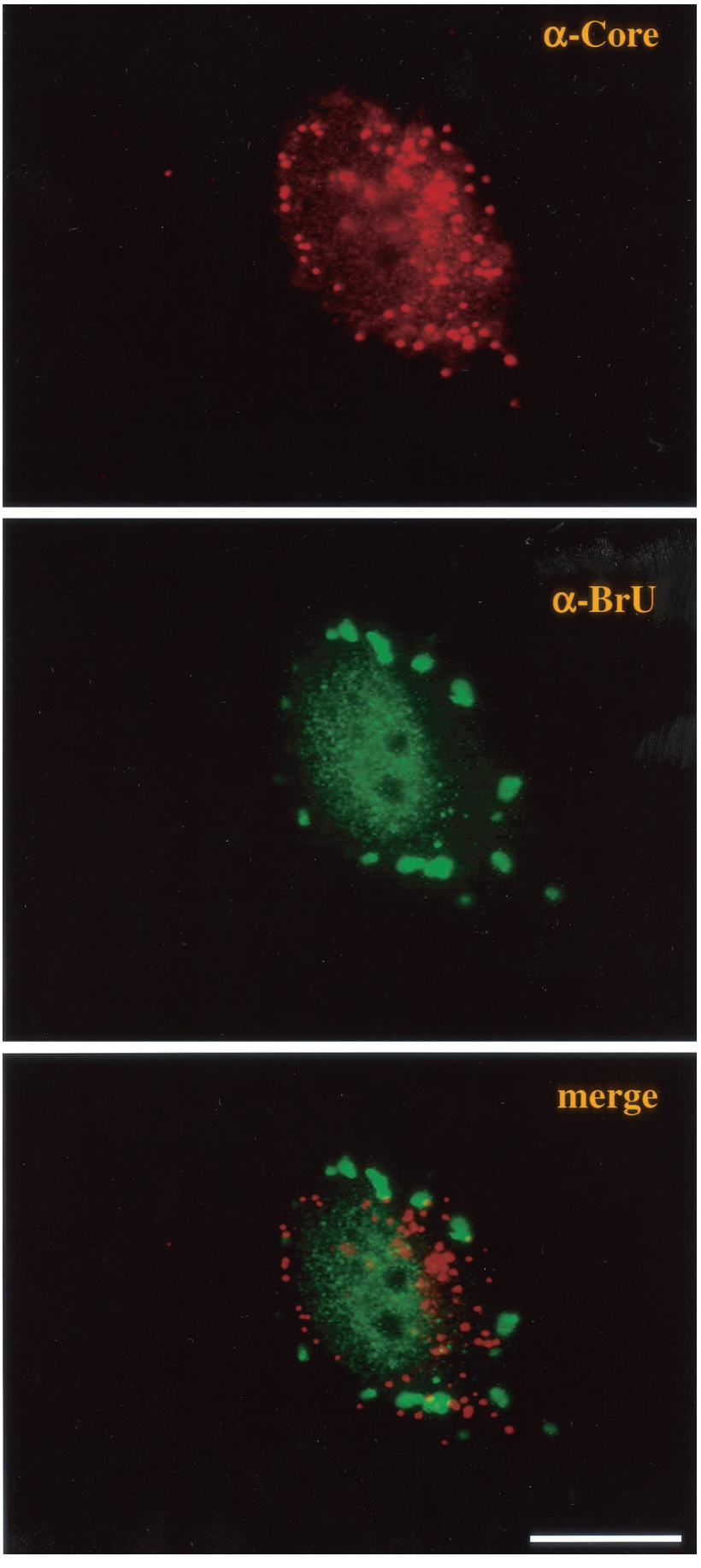
FIG. 2. Relation between MOI and the extents of early mRNA, DNA, and early protein synthesis. Cells were infected at the indicated MOIs as described in the legend to Fig. 1A and labeled either with [^{3}H]uridine or [^{35}S]methionine from 60 to 90 min postinfection or with [^{3}H]thymidine from 150 to 180 min postinfection. After the labeling period, cells were lysed; equal amounts of OD_{595} units, measured by a Bio-Rad protein assay, were precipitated with trichloroacetic acid; and the amount of radioactivity contained in each sample was determined by liquid scintillation counting. Values are averages ± standard errors of the means of duplicate samples and from three independent experiments. Since the values for the [^{35}S]methionine-labeled samples were much higher than those obtained for [^{3}H]uridine or [^{3}H]thymidine labeling, the [^{35}S]methionine values in the graph represent the real values divided by 10.

mulated some distance from intracellular cores. Both these observations opened up the possibility that the messengers were perhaps made at these sites and that (in contrast to what was assumed from the old literature) the viral genome, the template for transcription, might also be located at the RNA sites rather than remaining in the core. In the same study we also showed that both cores and mRNA structures associated with microtubules (MTs). Thus, an alternative explanation of our observations was that the messengers were made inside the cores, from which they were efficiently transported along MTs to their sites of accumulation and translation.

Because of these two possibilities we decided to monitor the fate of the parental DNA in a more direct way, by EM. We took advantage of the observation that inhibition of protein synthesis early in infection does not affect viral early transcription (15). Under the same conditions, the genome has been shown to remain insensitive to digestion with DNase, which has been interpreted to mean that the DNA remained inside the core (10, 12, 26). However, because of the indirect way of measuring uncoating, an alternative explanation, that the genome does leave the core before transcription but is coated with proteins that make it DNase resistant, cannot be excluded. In the present study we took advantage of the fact that inhibitors of protein synthesis blocked "uncoating" but not transcription to study the fate of the parental DNA under conditions of early mRNA synthesis.

Translation of the early messengers is required to initiate the process of DNA replication. The latter process has been shown to occur in discrete cytoplasmic structures that recruit a subset of VV early proteins (2, 3, 6, 16, 25, 29, 33). Transcription may also occur at these sites (5). Since the process of DNA replication induces a switch from early to late mRNA synthesis (4, 23; for reviews see references 21 and 22), it seems most logical to assume that transcription occurring on the sites of DNA replication results in the production of predominantly late transcripts (see also Discussion).

We have recently shown, in contrast to what was generally assumed, that the sites of DNA synthesis do not lie free in the cytoplasm. Instead, we found that from the earliest time point of VV DNA synthesis, starting around 2 h postinfection in HeLa cells, this process appeared to occur in close proximity to the cytosolic side of endoplasmic reticulum (ER) cisternae. As infection proceeded, more ER cisternae were apparently attracted to these sites, since about 45 min later the sites were almost completely enclosed by ER membranes (29). Several pieces of evidence suggested that the observed ER enclosure of the replication sites may promote efficient VV replication. For example, viral replication was low when the envelope formation was not completed early in infection but peaked when the sites were entirely ER enwrapped. After we established that the sites of mRNA accumulation and the DNA replication sites had rather unique morphological features, it was necessary to ask about their possible intracellular relationship. Synthesis of viral early proteins, which can be assumed to occur at the sites of mRNA accumulation, is required for initiation of DNA replication and for the biogenesis of these sites (reviewed in references 21 and 22). Considering this obvious molecular relationship, would the sites of DNA synthesis then


α-Core
α-BrU
merge

be initiated at the sites of mRNA accumulation, since the former would synthesize the molecules required for replication? What would be the fate of the mRNA structures once DNA synthesis was initiated?

The present study addresses the relationships between the three "early" intracellular structures that occur during the early phases of the VV life cycle: the intracellular core (and the fate of the parental DNA it contains), the sites where early mRNAs accumulate, and the sites of DNA synthesis. We first establish a quantitative relationship between incoming cores and the extents of mRNA and DNA synthesis. Then we visualize all three structures in infected cells by immunofluorescence microscopy (IF) and show that the sites of mRNA accumulation and of DNA synthesis are distinct structures that are located at different places. Finally, by EM we show that the parental DNA remains inside the core under conditions that allow transcription but that inhibit protein synthesis. When the parental DNA is able to leave the core, however, EM images strongly suggest that this happens in close proximity to the cytosolic side of membranes of the ER, where DNA synthesis, as we showed previously, is subsequently initiated.

MATERIALS AND METHODS

Cells, virus, and antibodies. HeLa cells were grown as described previously (28). The WR strain of VV was grown in monolayers of HeLa cells and semipurified as before (24). The virus was plaque titrated on BSC-40 cells. The following antibodies were used in this study: anti-core antibody (24), anti-p35 (H5R) (29), anti-BrU (Harlan Seralab, Loughborough, Leicestershire, United Kingdom), and anti-DNA (Roche Biochemicals, Mannheim, Germany). The donkey anti-rabbit antibody coupled to fluorescein isothiocyanate (FITC) or rhodamine and the goat anti-rat antibody coupled to FITC were from Jackson Immunochemicals (Dianova, Hamburg, Germany), and strepavidin conjugated to FITC or rhodamine was from Sigma (St. Louis, Mo.). 4′,6′-Diamidino-2-phenylindole (DAPI) was from Sigma and was used at 1 μg/ml for 20 min at room temperature.

Immunofluorescence and transfection of BrUTP or the biotinylated antisense H5R oligonucleotide. HeLa cells were grown on coverslips and infected as follows. Cells were washed once with prewarmed serum-free Dulbecco's modified Eagle medium (DMEM), and semipurified virus diluted in the same medium was briefly sonicated and applied to the cells for 15 min at 37°C. The cells were then washed with phosphate-buffered saline, incubated in serum-free DMEM, and fixed at the indicated times postinfection. Intracellular cores were visualized at 90 min postinfection, in the presence of 5 μg of cycloheximide/ml to block core degradation, by using the anti-core antibody. The sites of DNA replication were visualized by labeling cells fixed at 3 h postinfection, without inhibitor, with antibodies to p35 (H5R). Quantitation of intracellular cores and replication sites was performed on 30 cells and in three independent experiments. The average size of the DNA replication sites was estimated with NIH Image by measuring the diameter of each replication site in 10 cells. To visualize viral early mRNAs, cells were infected and transfected either with BrUTP or with an antisense oligonucleotide corresponding to the H5R mRNA as described elsewhere (19). Cells (1.5×10^5) were grown on 11-mm-diameter round coverslips in 24-well plates. They were washed twice in serum-free DMEM and infected with WR for 15 min at 37°C at a multiplicity of infection (MOI) of 50. After infection, cells were washed and lipofected (Lipofectin reagent; Gibco-BRL, Gaithersburg, Md.). Briefly, 10 mM 5′-bromouridine 5′-triphosphate (Sigma) or 2 μg of an antisense oligonucleotide corresponding to the H5R gene (2′ O-methylated; 5′-GCCAUCUUUGUGAAACUAGUAUC-3′) coupled to 4 biotin moieties was preincubated for 30 min in 30 μl of transfection medium containing 3.7 μl of Lipofectin. Samples were diluted in 300 μl of transfection medium and added to the cells in the presence of 5 μg of actinomycin D (actD)/ml to synchronize the incorporation of BrUTP or the H5R oligonucleotide into the viral mRNAs. After 1 h of incubation, cells were extensively washed, incubated in complete culture medium containing 5 mM hydroxyurea (HU; Sigma [unless otherwise indicated]), and fixed at the indicated times post-actD washout. HU was prepared as a 1 M stock in H_2O and kept at −20°C in aliquots. Once thawed, aliquots were discarded after use and not refrozen. Under these conditions the drug reproducibly blocked viral DNA replication, as assessed by the absence of Hoechst-positive replication sites in the cytoplasm of the infected cells.

HU washout experiment and double transfections. HeLa cells were grown on coverslips and infected as described above. To visualize early mRNAs, cells were lipofected with BrUTP or with the antisense oligonucleotide corresponding to H5R mRNA for 2 h in the presence of 2 mM HU. They were washed twice with serum-free medium and then fixed either immediately or 1 or 2 h after HU washout. In one experiment, after HU washout the cells were transfected with the H5R antisense oligonucleotide by hypotonic shift (Influx Pinocytic Cell-loading reagent; Molecular Probes) as described by the manufacturer and were fixed 2 h after the second transfection. Fixed cells were then either double labeled with anti-BrU and DAPI or triple labeled with anti-BrU (followed by an anti-rat antibody coupled to rhodamine), DAPI, and streptavidin-FITC.

Metabolic labeling. Monolayers of HeLa cells were infected at the indicated MOI and labeled with 50 μCi of [^{3}H]uridine or [^{35}S]methionine/ml from 60 to 90 min postinfection or with 50 μCi of [^{3}H]thymidine/ml from 2 h 30 min to 3 h postinfection. Cell lysates were prepared as described by Mallardo et al. (19). Briefly, after the labeling period, cells were washed and collected in cold phosphate-buffered saline in Eppendorf tubes. Cells were lysed in lysis buffer (50 mM HEPES [pH 6.9], 100 mM KCl, 1 mM dithiothreitol, and 0.5% Nonidet P-40), and the amount of protein was measured by a Bio-Rad protein-assay. For uridine incorporation, 100 U of RNase inhibitor (Promega)/ml was added to the lysis buffer. Equal amounts of OD_{595} (optical density at 595 nm) units were precipitated with trichloroacetic acid and counted by liquid scintillation counting. Values are averages from duplicate samples and from three independent experiments.

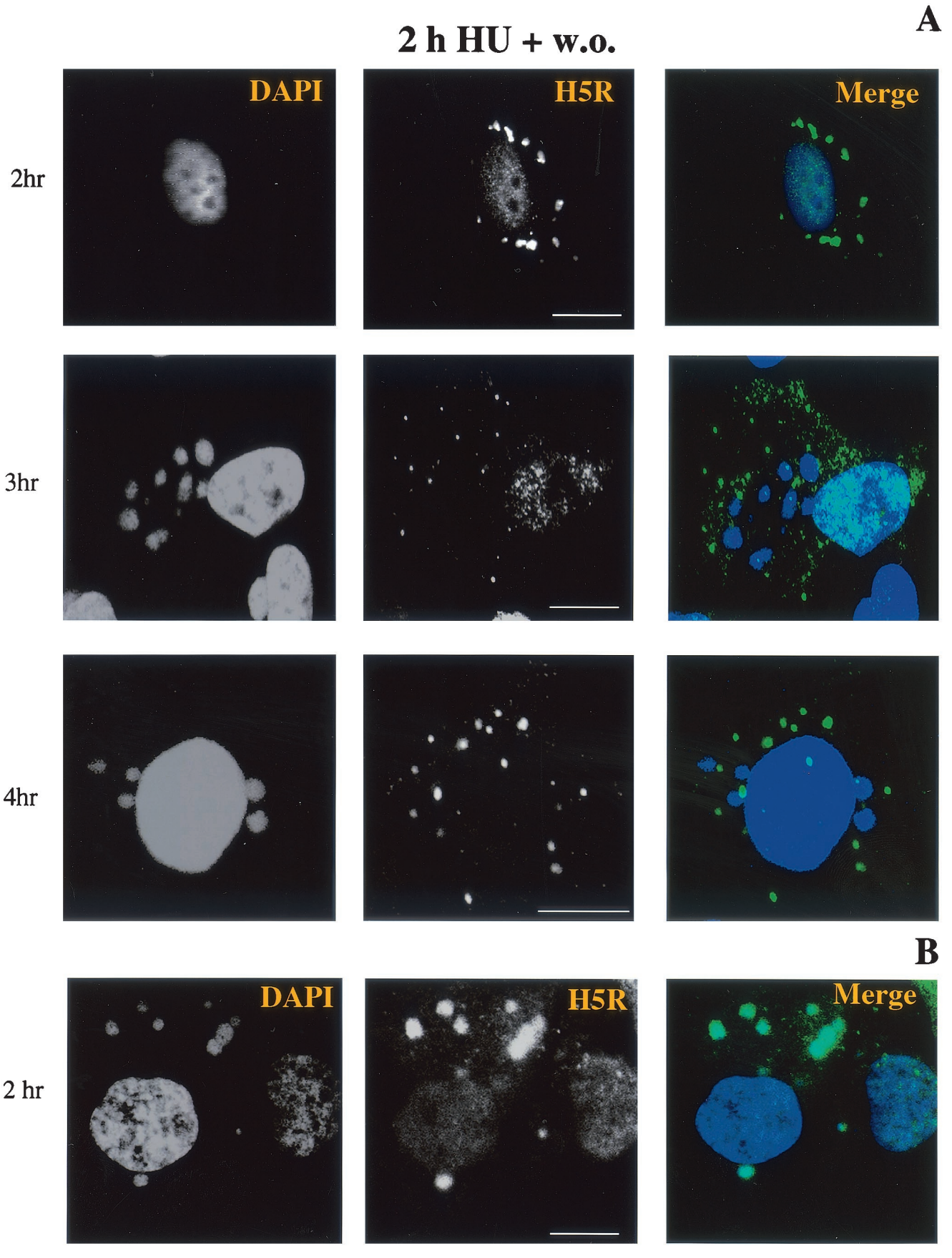
EM. Monolayers of HeLa cells were infected at an MOI of 200 in the presence or absence of 1 μg of actD (Sigma)/ml, 5 μg of cycloheximide (Sigma)/ml, or 5 mM HU (Sigma). Cells were fixed at 60 and 120 min postinfection and processed for cryosectioning as described previously (30). Quantitation was performed by counting the gold particles associated with 20 intracellular cores.

RESULTS

Quantitative relationship between the number of intracellular cores and early mRNA and DNA synthesis. In several of our recent publications we have described a number of assays to monitor VV intracellular cores, the intracellular sites of VV early mRNA accumulation, and the sites of VV DNA synthesis (18, 19, 29). Intracellular cores can be visualized by IF using an antibody raised against VV cores. By light microscopy this antibody recognizes intracellular cores only and not extracellular virions (18). The sites where VV early mRNAs accumulate can be detected by transfecting infected cells either with BrUTP or with a biotinylated antisense oligonucleotide corresponding to the H5R mRNA, an abundant early/late VV gene. VV DNA synthesis is known to occur in distinct cytoplasmic sites (2, 3, 16). To visualize these sites, infected cells were labeled either with DAPI or with an antibody to H5R (p35), since this protein colocalizes with the VV replication sites as soon as DNA synthesis starts (2, 29).

These assays enabled us to determine the quantitative rela-

FIG. 3. Intracellular cores and the sites of early mRNA accumulation do not colocalize. HeLa cells grown on coverslips were infected and transfected with BrUTP as described in Materials and Methods in the presence of 5 mM HU in order to block DNA replication. Cells were fixed at 2 h postinfection and double labeled with the anti-core antibody, followed by donkey anti-rabbit coupled to rhodamine, and anti-BrU, followed by goat anti-rat–FITC. The merge in the bottom panel shows that the two structures do not overlap at all. Bar, 10 μm.


A
2 h HU + w.o.
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2hr
3hr
4hr
B
DAPI
H5R
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tionship between cores, mRNA sites, and DNA sites by light microscopy. Cells were infected with increasing MOIs, and intracellular cores were counted at 90 min postinfection. To synchronize the infection, cells were infected for 15 min at 37°C, after which excess virus was removed by washing. Because the entry of the intracellular mature virus (IMV) is rather slow, this short adsorption time required relatively high MOIs in order to obtain significant amounts of intracellular cores. We have previously shown, for instance, that under different infection conditions (binding of the IMV for 60 min at room temperature followed by entry at 37°C) and at an MOI of 50, about 30 cores are able to enter HeLa cells after 60 min at 37°C. At the same MOI under the same conditions, the number of intracellular cores was less than 5 after 15 min of penetration (18). Our present experimental setup differed from our previously published protocol in that the absorption time was limited to 15 min at 37°C, after which intracellular cores were counted at 90 min postinfection in the presence of cycloheximide to block core degradation. With such a relatively short absorption time, we found that an MOI of 100 was required to obtain an average of 20 cores per cell at 90 min postinfection, while an MOI of 1,600 resulted in an average of 130 cores (Fig. 1A).

Figure 1A shows that the number of intracellular cores increased linearly with increasing MOI. The number of DNA replication sites, visualized by using anti-p35 labeling at 3 h postinfection, appeared to follow a similar pattern initially, and at the three lowest MOIs used, their number increased in a linear fashion. Above 40 cores per cell, however, the number of replication sites did not increase further. The extent of DNA synthesis, however, increased with increasing MOI, as shown by measuring the average size of the DNA sites at 3 h postinfection. We found that their average size increased linearly with increasing MOI, suggesting that the extent of DNA synthesis increased as more cores were able to enter the cells (Fig. 1B) (see also below). The fact that the number of replication sites did not increase beyond an average of 40 cores per cell can be explained in two ways. Above 40 cores per cell, the genomes of more than 1 core started to contribute to the generation of 1 (seemingly larger) replication site. Alternatively, if replication sites were numerous in the cell, they tended to merge into several large sites.

Attempts to quantify the number of VV mRNA sites relative to intracellular cores in a similar manner were unsuccessful; we found that as the MOI increased, the viral mRNAs became less organized into discrete granular structures (see below and reference 19). Instead, at higher MOIs, the mRNAs showed both an organized and a general diffuse pattern in the cytoplasm of infected or BrUTP-transfected cells, which made it difficult to reliably count their numbers (see Discussion). Therefore, the relationship between early mRNA synthesis and incoming cores was determined biochemically. Cores were counted at 90 min postinfection by IF, and a parallel set of cells, infected at the same MOI, was labeled either with [^{3}H]uridine or with [^{35}S]methionine (see below) from 60 to 90 min postinfection for viral early mRNAs or proteins, respectively, or with [^{3}H]thymidine from 2 hr 30 min to 3 h postinfection for labeling of viral DNA. We have recently shown that in our HeLa cells, DNA synthesis is not initiated until 2 h postinfection (29), and therefore these labeling times would be expected to coincide with peaks in early mRNA or DNA synthesis, respectively. Figure 2 shows that the incorporation of [^{3}H]uridine increased almost linearly with increasing MOI, but leveled off at the highest MOI used. The increase in DNA synthesis followed a similar pattern (an almost-linear increase, followed by leveling off at the highest MOI), although the extent of [^{3}H]thymidine incorporation was lower than that of [^{3}H]uridine incorporation. For both uridine and thymidine incorporation there appeared to be no increase (rather a slight decrease) at the two lowest MOIs used. This was most likely a reflection of the fact that at MOIs of 50 and 100 (using an adsorption time of 15 min at 37°C), not all cells were infected.

The above data suggested that although the VV mRNAs were less organized at high MOIs, this did not affect the extent of mRNA synthesis. We wondered, however, whether the failure of the mRNAs to organize into their typical granular structures at high MOIs could affect their translation. For this, cells infected at increasing MOIs were labeled with [^{35}S]methionine from 60 to 90 min postinfection to label early proteins. Indeed, as the MOI increased, [^{35}S]methionine incorporation increased at the three lowest MOIs used, but above an MOI resulting in more than 40 cores per cell, the extent of protein synthesis appeared to level off and even to decrease slightly. These data may suggest that the partial failure of mRNAs to organize at high MOIs affected efficient (VV early) protein synthesis.

The combined data show that there is a linear relationship between the MOI, the number of intracellular cores, and the extents of RNA and DNA synthesis. They show that at MOIs resulting in 10 to 40 cores per cell, there is a ratio of approximately 1:1 between cores and DNA replication sites, suggesting that each incoming core is able to generate a site of DNA synthesis. The latter results are consistent with the findings of Cairns (3) showing that one infectious particle is able to generate one site of viral replication.

Cores and sites where early mRNAs accumulate are distinct. We have recently shown that the cytoplasmic sites where VV early mRNA accumulated, visualized by using BrUTP (or a

FIG. 4. DNA replication may not be initiated where the early mRNAs accumulate. (A) Monolayers of cells grown on coverslips were infected and lipofected with a biotinylated antisense oligonucleotide corresponding to H5R mRNA as described in Materials and Methods in the presence of 2 mM HU. At 2 h postinfection, the HU was washed out and cells were incubated for an additional 1 and 2 h before being fixed and double labeled with DAPI and streptavidin-FITC (to detect the H5R oligonucleotide). The times postinfection are given on the left: 2 h postinfection corresponds to the time of HU washout, and 3 and 4 h postinfection correspond to 1 and 2 h post-HU washout, respectively. Note the absence of DAPI-positive replication sites at 2 h and the noncolocalization of the DAPI and H5R labels at 3 and 4 h. (B) Cells were infected for 2 h in the presence of HU, and the drug was then washed out, followed by transfection with the biotinylated H5R oligonucleotide. Cells were fixed at 2 h posttransfection (indicated on the left) and double labeled with DAPI and streptavidin-FITC (H5R). Note that under these conditions of infection and transfection, the DAPI- and streptavidin labels largely overlap. Bars, 10 μm.

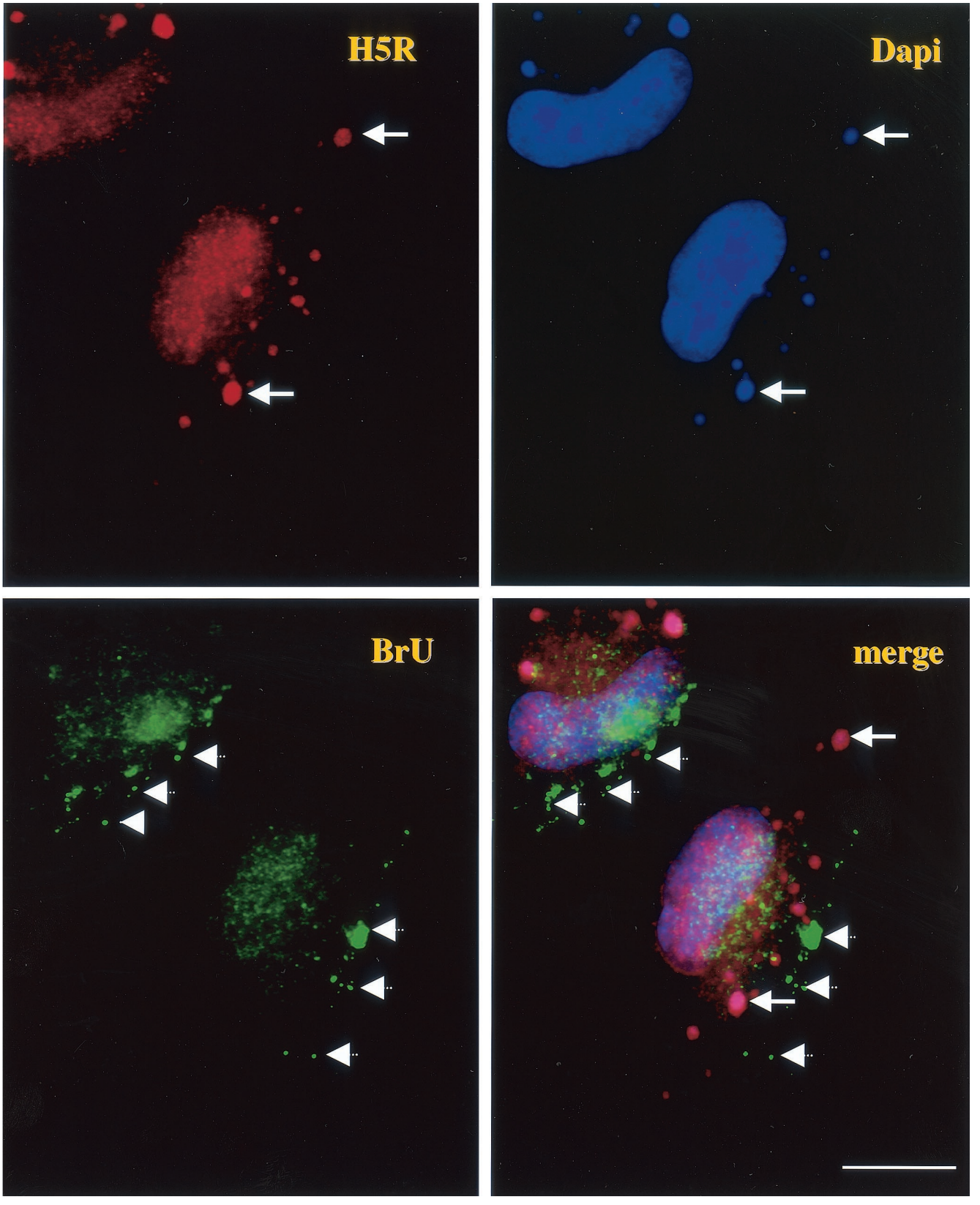


FIG. 5. Double transfection of BrUTP and the antisense H5R oligonucleotide at different times of infection reveals two different structures that accumulate viral mRNAs in infected cells. HeLa cells were infected and transfected with BrUTP for 2 h in the presence of 2 mM HU. HU was washed out, and cells were transfected with the antisense oligonucleotide corresponding to H5R mRNA. Double-transfected cells were fixed 2 h after HU washout and triple labeled with anti-BrU (FITC) (yellow channel), streptavidin-rhodamine (H5R) (red channel), and DAPI (blue

biotinylated antisense oligonucleotide corresponding to H5R mRNA) transfection, were always located some distance from intracellular cores, and we have provided evidence showing that both structures were aligned on MTs (19).

For the sake of clarity in the experiments that follow below, we first confirmed part of these observations in the present study. HeLa cells were infected and transfected with BrUTP in the presence of actD. Subsequently, actD was washed out to allow synchronized incorporation of BrUTP into viral mRNAs. Infected and transfected cells were fixed at 2 h postwashout in the presence of HU to block DNA replication (in order to study the process of early transcription only, independent of the subsequent stages of infection) and were double labeled with anti-BrU and the anti-core antibody. The anti-core antibody detected punctate structures that we recognized as intracellular cores and that appeared randomly scattered in the cytoplasm (Fig. 3). Anti-BrU, however, labeled distinct structures, about 1 μm in diameter, that were not obviously located next to cores (Fig. 3). Using BrUTP transfection as described above, we have shown before that BrU labeling in the cytoplasm of infected cells corresponded to sites where viral early messengers collect. No such labeling was observed in uninfected cells. Moreover, upon cotransfection of infected cells with BrUTP and a biotinylated antisense oligonucleotide corresponding to H5R mRNA, an abundant early/late gene, the labeling for the two probes completely overlapped (19).

Having confirmed our recent results showing that the early mRNAs generally accumulate in structures that are distinct from intracellular cores, we next wondered about the intracellular relationship between the DNA replication sites and the early mRNAs. As reported below, a series of experiments was conducted that showed that these also represented distinct structures.

The DNA replication sites are not initiated where early mRNAs accumulate. In the study by Mallardo et al. (19), we observed that the sites where the early messengers accumulated in the cytoplasm appeared to grow in size over the time of infection. As explained in the introduction, this observation opened up the possibility that transcription occurred at these sites and that thus the parental DNA might also be located at these sites. If the latter were true, we expected DNA replication to start at the early mRNA sites, since these contained the parental DNA. This possibility was tested in the following way. Cells were infected and transfected with the biotinylated antisense oligonucleotide corresponding to H5R mRNA (19) in the presence of low concentrations of HU to block viral DNA synthesis. At 2 h postinfection, HU was washed out, and cells were fixed at 0, 1, and 2 h postwashout. They were subsequently double labeled with streptavidin-FITC to visualize early mRNAs and with DAPI to label the sites of viral DNA synthesis. If DNA synthesis was indeed initiated at the sites of mRNA accumulation, we expected the two labels to overlap significantly. The cells fixed at the time of HU washout showed the typical granular structures representing early mRNAs, while no DAPI-positive sites were yet to be seen (Fig. 4A, top). At 1 and 2 h after HU washout, however, DAPI-positive replication sites could easily be detected, and they did not appear to overlap or to lie next to the mRNA sites (Fig. 4A). This difference was best appreciated at 2 h post-HU washout, when the mRNAs were organized into discrete spots that were clearly localized to cytoplasmic sites different from the DNA replication sites (Fig. 4A).

H5R mRNA, encoding an early/late protein (17), can be expected to be transcribed early as well as late in infection. It was therefore somewhat surprising that under the experimental setup described above, no H5R transcription was detected on the sites of DNA replication. As mentioned in the introduction, once DNA replication has been initiated, the DNA sites may become the (major) sites of viral (intermediate and late) transcription (see reference 5). By performing a detailed time course of BrU or H5R incorporation in the absence of HU, we found that both probes were efficiently incorporated into the early mRNA structures but never labeled the DNA replication sites that appeared later in infection (data not shown). Apparently, upon transfection of BrUTP or the H5R oligonucleotide from the beginning of the infection, both probes were entirely incorporated into early mRNAs extruded from the cores, such that when DNA replication was initiated 2 h later, they were no longer available for incorporation into late mRNAs.

To demonstrate that transcription of H5R could also occur on the sites of DNA replication, cells were infected for 2 h in the presence of HU, after which the drug was washed out and cells were transfected with the H5R oligonucleotide and fixed 2 h later. Under these conditions the bulk of the H5R mRNA labeling now clearly colocalized with the DAPI-positive replication sites (Fig. 4B). Similar results were obtained when BrUTP was used instead of the H5R oligonucleotide (data not shown).

The data thus suggested the existence of two distinct structures involved in the transcription of mRNAs: one in which early mRNAs apparently accumulated and one involving transcription on the sites of DNA replication. Furthermore, our HU washout experiment suggested that these were distinct structures, located at distinct sites in the cytoplasm. This point was further investigated below.

Double-transfection experiments reveal two distinct structures that accumulate mRNAs in infected cells. The above observations implied that by transfecting with either BrUTP or the H5R oligonucleotide from the beginning of the infection or only after DNA replication had started, mRNAs could be detected at two different cytoplasmic sites. This observation was next confirmed by double-transfection experiments.

Cells were infected and transfected with BrUTP in the presence of low concentrations of HU to block DNA synthesis and to label early mRNAs. Subsequently, HU was washed out, and

channel). The solid arrows in all four panels indicate DAPI-positive replication sites, the labeling of which entirely colocalizes with the pattern of the H5R oligonucleotide. The BrU pattern, indicated by hatched arrows (BrU and merge panels), does not colocalize with either of the two labels. It accumulates in granular structures that appear to have no relationship with the replication sites. This is most dramatically shown in the upper left cell, in which the replication sites all seem to collect at the upper left side of the cell, while the BrU-positive structures accumulate at the lower right side of the same cell. Bar, 10 μm.

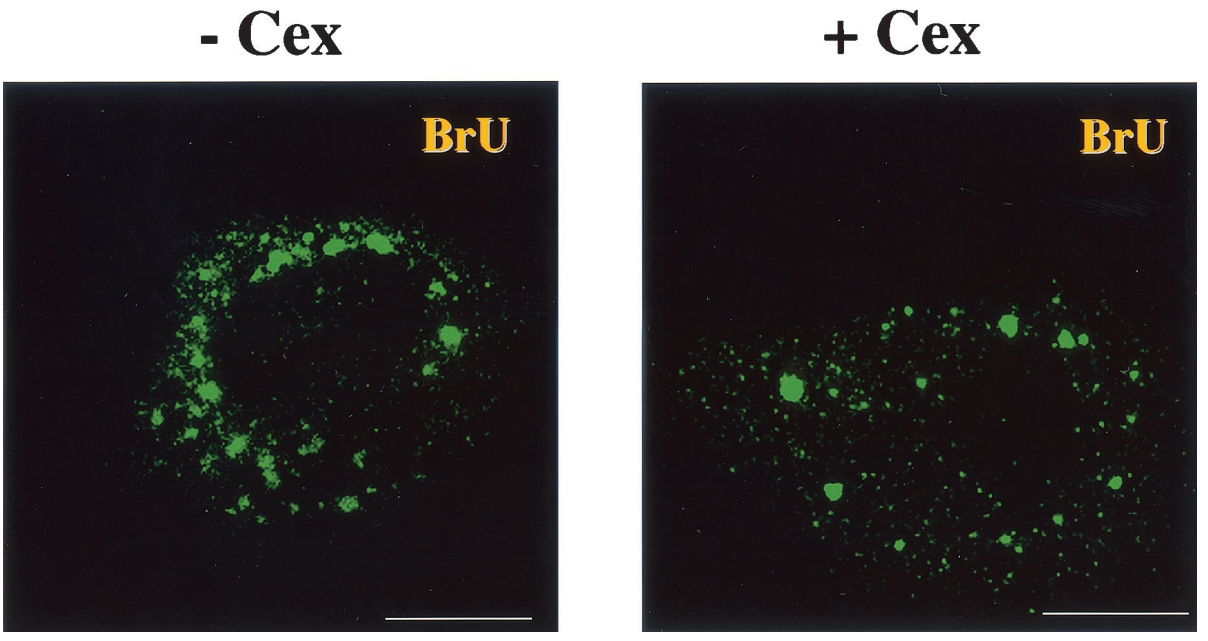


FIG. 6. The sites of mRNA accumulation develop in the same way in the presence of cycloheximide as in the absence of the inhibitor. HeLa cells were infected and transfected with BrUTP as described previously (19) in the absence (− Cex) or presence (+ Cex) of 5 μg of cycloheximide/ml. Infected cells were fixed at 2 h postinfection and labeled with anti-BrU. Bars, 10 μm.

cells were transfected with the biotinylated oligonucleotide corresponding to H5R mRNA at the time of washout and were fixed 2 h later. Fixed cells were triple labeled with anti-BrU, streptavidin-FITC (to reveal the H5R oligonucleotide), and DAPI (to visualize the replication sites). Since the H5R oligonucleotide was transfected after replication was initiated, it completely colocalized with the sites of DNA replication, in agreement with the above results (Fig. 5). The BrU label, however, which reflected BrUTP transfection performed immediately after infection, did not colocalize with the replication sites at all. The BrU label appeared as typical granular structures, which we have shown before to correspond to the sites of mRNA accumulation, that did not seem to correspond to the sites of DNA synthesis; they bore no obvious relation to them and appeared scattered over the cytoplasm, not obviously close to the DAPI-positive sites. In some instances they even seemed to accumulate in areas of the cell entirely different from those where the replication sites were found (Fig. 5). Identical results were obtained when the H5R oligonucleotide was transfected first, followed by transfection of BrUTP after HU washout (data not shown).

Thus, the data strongly suggest that the sites where early mRNAs accumulate and those involved in DNA synthesis are distinct structures. Furthermore, our data suggested that the process of DNA replication is not initiated where the early mRNAs concentrate. Indirectly, the latter results also imply that mRNA synthesis occurs inside the core and that the parental DNA remains inside the core until uncoating and subsequent DNA synthesis start. This point was addressed in more detail by EM.

The fate of parental DNA as assessed by EM. When cells are infected in the presence of the transcriptional inhibitor actD, intracellular cores that contain the viral genome accumulate, as observed by EM (24). It is generally assumed that protein synthesis is required to uncoat the parental DNA, since inhibition of protein synthesis early in infection blocks this process (see the introduction). It has, however, been shown biochemically that inhibition of protein synthesis does not interfere with the process of early transcription (15).

To confirm that the early mRNAs collected in similar granular structures in the presence of cycloheximide, we repeated the experiment for which results are shown in Fig. 3 and infected and transfected HeLa cells with BrUTP, both with and without the protein synthesis inhibitor (in the presence of HU to block DNA synthesis). Indeed, in the presence of cycloheximide, the BrU-labeled granular mRNA structures looked indistinguishable from those without the drug (Fig. 6) and organized some distance away from the cores (data not shown).

We then determined the location of the parental DNA in cycloheximide-treated cells; if the DNA remained inside the core under conditions in which transcription was not inhibited, it followed that this process must occur inside the core, from which the mRNAs were subsequently extruded to become organized into the typical granular structures (see the introduction).

Cells were infected in the presence of either actD (to confirm previous results [24]), cycloheximide to block protein synthesis, and HU to block DNA synthesis. It has been shown that the core uncoats under the latter condition (10), but since HU prevents viral DNA replication (see also Materials and Methods for the use of HU), it enabled us to monitor the fate of the parental DNA once it had left the core, by using EM (see below).

Cryosections of infected cells treated with the three drugs were labeled with anti-DNA. Figure 7 shows a few examples of such cryosections; it was clear that in the presence of cyclo-

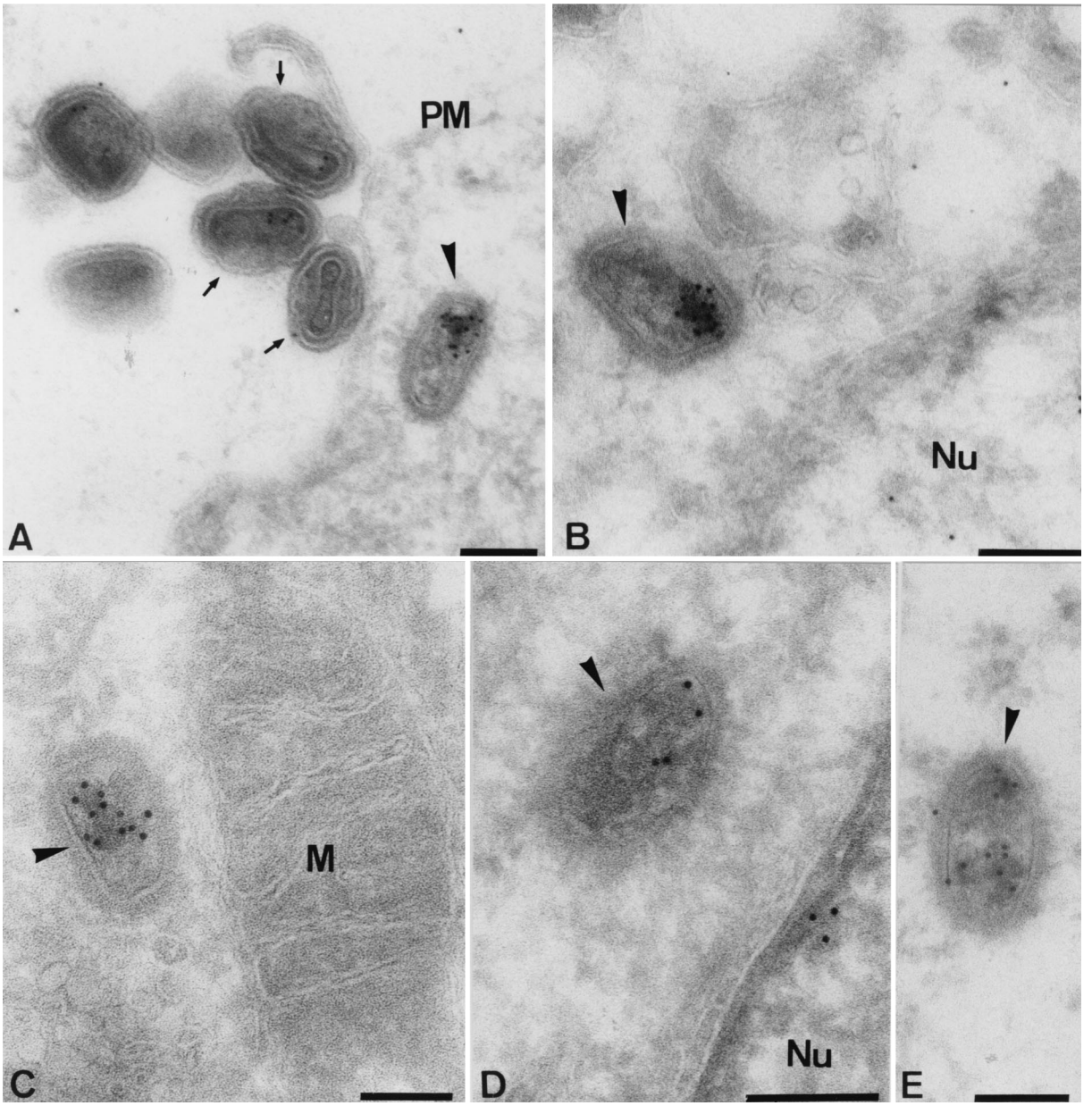


FIG. 7. Fate of the parental DNA under different conditions of infection. Monolayers of cells were infected at an MOI of 200 for 30 min at 37°C in the presence of either cycloheximide (A, B, and E), actD (C), or HU (D) and were fixed at 60 min (B and C) or 120 min (A, D, and E) postinfection. Cells were prepared for cryosectioning and labeled with anti-DNA. (A) Virions at the plasma membrane (PM) (small arrows) with low levels of label. One intracellular core (arrowhead) is clearly labeled. (B) A core close to the nucleus (Nu). (C) An intracellular core is found close to a mitochondrion (M). (D) The core accumulated in the presence of HU is less intensely labeled than the cores in the other images. (E) A core accumulated for 120 min in the presence of cycloheximide. Bars, 200 nm.

heximide, the genome remained inside the core, as in cells treated with actD. In the presence of HU, however, core profiles were much more difficult to find, suggesting that they were gradually degraded. In the remaining core profiles, the anti-DNA labeling appeared to decrease over time, consistent with the idea that the genome was able to leave the core.

To obtain a quantitative impression of the above morphological results, the average level of gold labeling per core at 60 and 120 min postinfection under the three different conditions was determined. The results are represented in Table 1, showing that both in the presence of actD and in the presence of cycloheximide, the average labeling on intracellular cores did not decrease over time. Instead, in the presence of actD, we observed a slight increase in anti-DNA labeling on the intracellular cores over time. While we are not sure that the latter increase in labeling is significant, a clear increase in labeling was observed in the presence of cycloheximide (an average of 7 gold particles/core at 60 min and 11.9 at 120 min). It appeared that some structural changes occurred inside the core that resulted in increased accessibility of the antibody to the

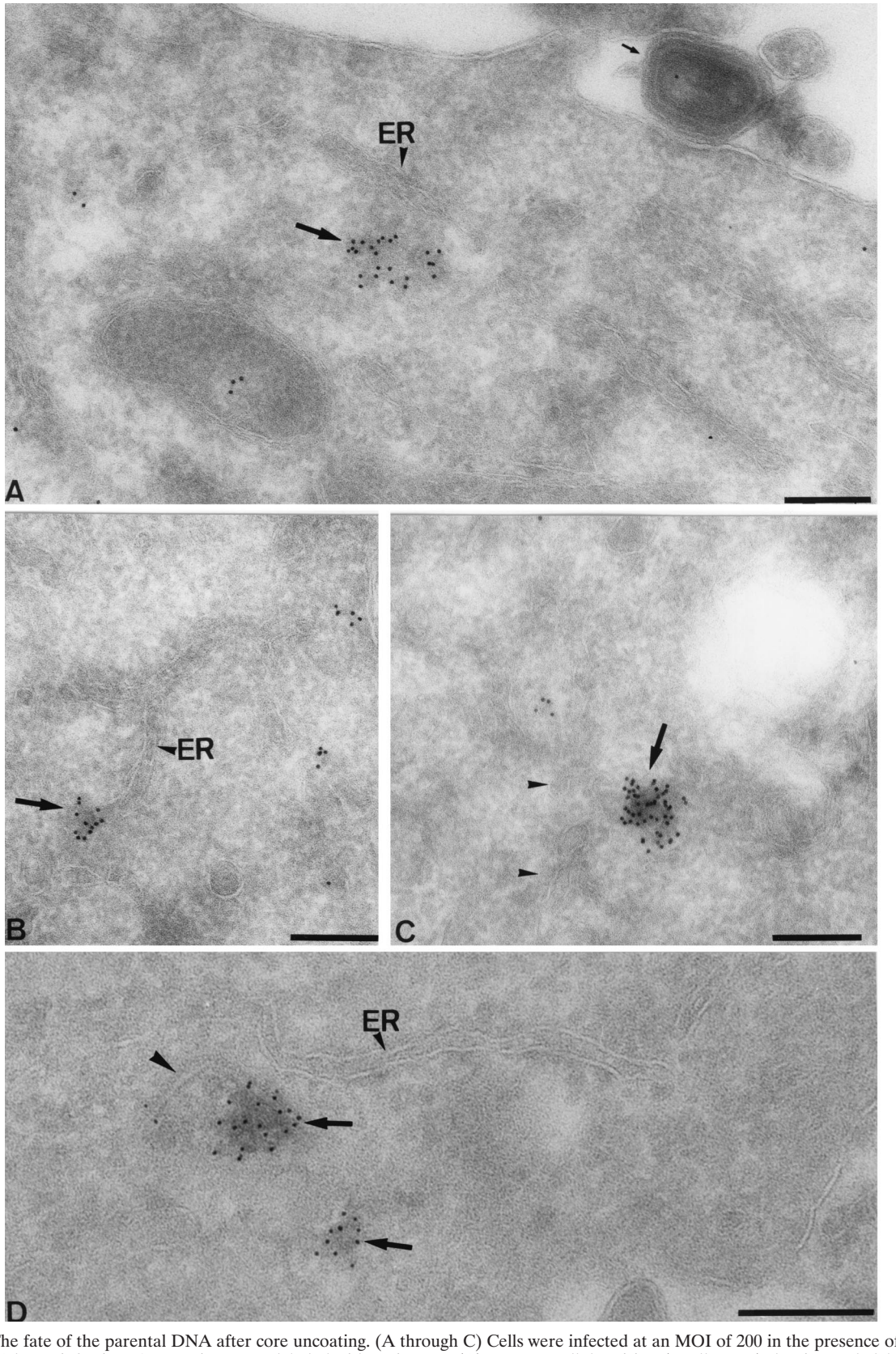


FIG. 8. The fate of the parental DNA after core uncoating. (A through C) Cells were infected at an MOI of 200 in the presence of 5 mM HU and fixed at 2 h postinfection. Cryosections were labeled with anti-DNA. (A) An extracellular virion (small arrow) that is poorly labeled. In the cytoplasm one area (large arrow) can be seen that is heavily labeled and that seems associated with the ER (arrowhead). (B) The ER (indicated) seems to be labeled with anti-DNA at three positions, one of which is indicated by a large arrow. (C) A cytoplasmic labeled patch (arrow) is found close to ER membranes (arrowheads). (D) Cells infected for 1 h without HU. The image shows a core that is in the process of uncoating (large arrowhead) close to the ER (small arrowhead). DNA labeling is associated with the disassembling core as well as with the surrounding cytoplasm (large arrows). Bars, 200 nm.

viral genome. As mentioned above, in the presence of HU, cores were generally more difficult to find. While the fact that cores were obviously being degraded in the presence of HU could bias the average level of gold labeling per core, the number of gold particles in the remaining core profiles showed a tendency to decrease. At 60 min postinfection the average level of labeling per core was lower than in the presence of actD or cycloheximide, and this number was even lower at 120 min, suggesting that over time the genome left the core under these conditions.

In conclusion, these data confirm that protein synthesis early in infection is required to release the parental DNA from the intracellular core.

The fate of the parental DNA after core uncoating. Next, we wanted to address the fate of the parental DNA once it had left the core. Cryosections of cells infected in the presence of HU and fixed at 2 h postinfection were prepared and labeled with anti-DNA (see above). As mentioned, cores were difficult to find in such cryosections, presumably because they were being degraded. Cytoplasmic anti-DNA labeling, seemingly attached to membranes of the ER or in close proximity to them, was seen (Fig. 8A through C). To ensure that this observation was not an artifact of HU treatment, we also labeled cryosections prepared from cells infected for 1 h without the inhibitor. Although the possibility that under these conditions viral DNA synthesis had started cannot be excluded, we considered this possibility unlikely. Our recent study showed that DNA replication in our HeLa cells is not initiated before 2 h postinfection (29). Under these conditions, cores were much easier to find and could be observed in what looked like different stages of uncoating. Cores were seen that apparently had not yet released their genome, since they were labeled with anti-DNA, while no labeling was found in the surrounding cytoplasm (Fig. 9A). "Empty" cores devoid of anti-DNA labeling, as well as cores that were labeled to some extent, were also seen. Some of the less intensely labeled or unlabeled cores gave the impression of having opened up on one side (Fig. 8D and 9B).

In a previous study, in which cores were accumulated in the presence of actD (24), we noticed that the cores were often found close to membranes of the ER, as seen by EM. At that time we had no explanation for this observation. In the present study cores that were apparently in the process of releasing the DNA were also found to accumulate close to the ER. Intact cores could be found close to these membranes, as could cores that had apparently started to release their DNA or had done so already. Strikingly, when core profiles that were less intensely labeled with anti-DNA, or not labeled at all, were found, the cytosolic side of the ER appeared to be labeled (Fig. 8D and 9B and C).

Thus, it seems that upon leaving the core, the parental DNA and/or its associated proteins have the intrinsic property of associating with membranes of the ER (see Discussion).

TABLE 1. Average anti-DNA labeling per intracellular core, per extracellular virus, and around the intracellular core

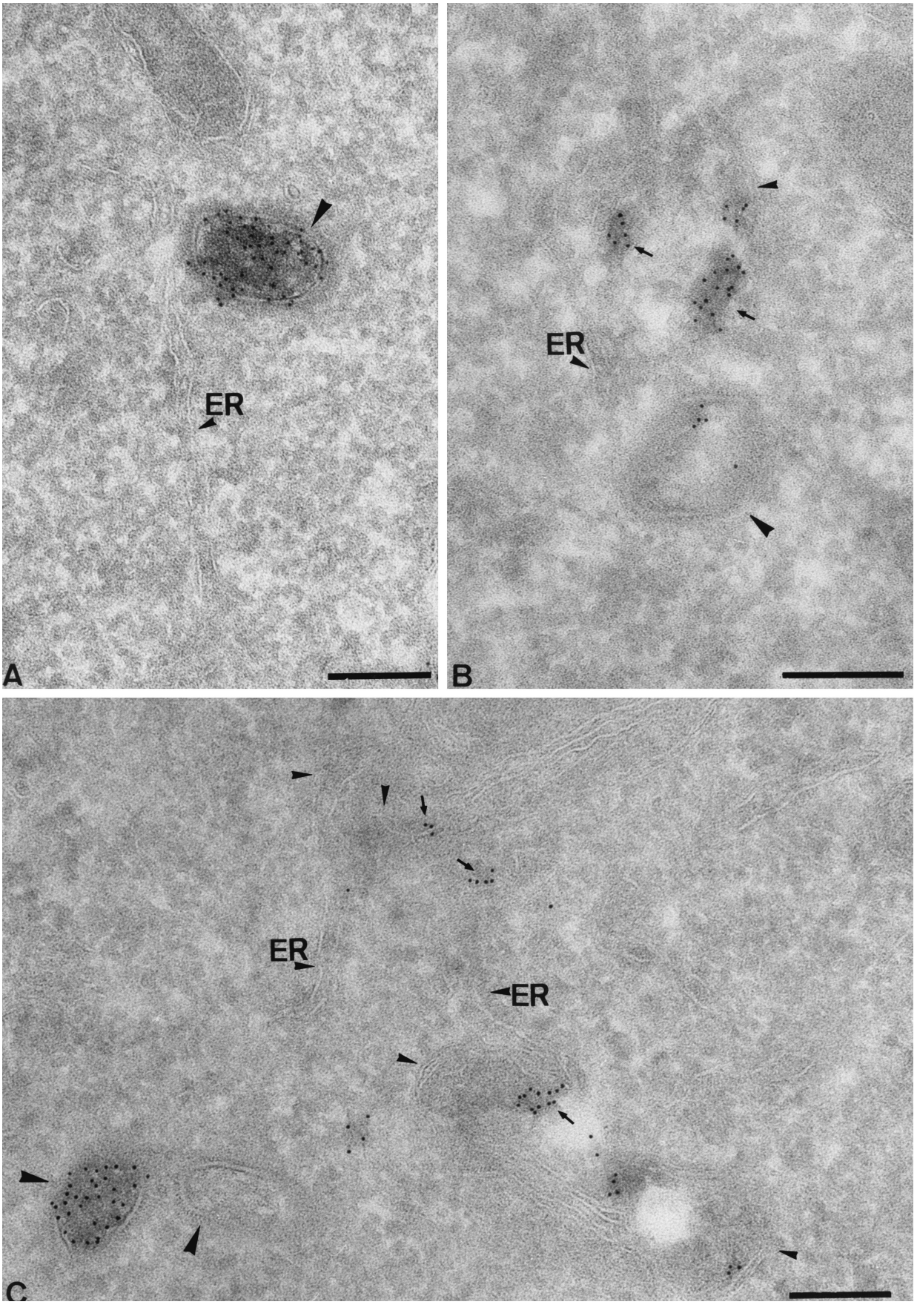
Infection condition[a]	No. of gold particles (avg ± SEM):		
	Per core[b]	Around core[c]	Per IMV[d]
actD, 60 min	4.6 ± 0.7	0.1 ± 0.01	1.1 ± 0.3
actD, 120 min	5.5 ± 0.8	0.6 ± 0.2	1.3 ± 0.4
CX, 60 min	7.0 ± 0.7	0.4 ± 0.2	1.0 ± 0.3
CX, 120 min	11.9 ± 1.6	0.8 ± 0.4	0.9 ± 0.3
HU, 60 min	2.9 ± 0.7	0.9 ± 0.3	1.3 ± 0.4
HU, 120 min	1.2 ± 0.6	1.3 ± 0.5	1.4 ± 0.4

[a] Cells were infected at an MOI of 200 for the indicated time (60 or 120 min) in the presence of either 1 μg of actD/ml, 5 μg of cycloheximide (CX)/ml, or 5 mM HU.
[b] Average number of gold particles on 20 intracellular cores.
[c] Average number of gold particles at a distance of 500 nm from 20 intracellular cores.
[d] Average number of gold particles on 20 extracellular IMVs. Note that the extracellular virions are generally poorly labeled with anti-DNA. This is mostly likely because the genome is poorly accessible to antibody labeling on cryosections in intact virions.

DISCUSSION

VV assembly, a late stage in infection that has been characterized in some detail biochemically, genetically, and morphologically (reviewed in reference 27), is preceded by a number of discrete steps that all occur in the cytoplasm of the infected host cell. The purpose of the present study was to investigate possible quantitative and qualitative relationships between intracellular cores and the parental DNA, the sites where VV early mRNAs accumulate and are translated, and the sites of DNA replication. An antibody that recognizes intracellular cores by IF enabled us to quantify incoming cores at a given MOI. Moreover, using this antibody, we established for the first time a quantitative relationship between incoming cores and replication sites. Our data show that at appropriate MOIs a 1:1 ratio between incoming cores and sites of DNA synthesis exists, implying that the genome of 1 core is able to generate 1 replication site and that thus each incoming core is "infectious." Furthermore, we show that when the numbers of incoming cores increased, the extents of RNA and DNA synthesis increased in a linear fashion. Our data show that cores, the mRNA sites, and the DNA sites are distinct structures that, unexpectedly, do not obviously lie next to each other in the cytoplasm. Finally, our data reveal that the cellular ER is intimately involved with both cores and the parental DNA. These data thus extend the role of this organelle to the earliest stages of the VV life cycle.

The genome remains inside the core during transcription. As outlined in the introduction, we have shown before that VV early mRNAs accumulate in distinct structures that assemble some distance away from intracellular cores. We felt that, for the sake of clarity, we had to include a similar experiment in the present study, to unequivocally demonstrate that cores and early mRNAs are clearly two distinct intracellular structures that are not obviously connected. In our previous study we discussed two possibilities to explain the fact that the mRNA sites also appeared to grow over time. Either the genome moved to the RNA sites during the process of transcription and at least some of the early mRNAs were made at these sites or, alternatively, since both the mRNA structures and the cores were shown to be associated with MTs, mRNAs were efficiently transported along MTs from their sites of synthesis inside the cores to the sites of accumulation (see the introduction). The present study shows two pieces of evidence that favor the latter possibility. First, we show directly, by EM, that the viral genome remains inside the core during transcription. Inhibition of protein synthesis early in infection appeared to


ER
A
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prevent the genome from leaving the core, as shown biochemically before (15). Second, DNA synthesis does not appear to be initiated at the sites where the mRNAs accumulated. If the genome is located at the mRNA sites, DNA replication can be expected to start at these sites as well. Thus, it seems that, as in vitro, early mRNAs are made inside the core, from which they are subsequently extruded. It is not clear why, once extruded, the early mRNAs always seem to collect some distance away from intracellular cores and not, as might seem more logical, next to the sites of their synthesis. Perhaps the subsequent process of DNA replication, which, as we argue below, may start at the site where the core releases the parental DNA, precludes the accumulation of another rather large structure next to this structure.

The sites of early mRNA accumulation and DNA synthesis are distinct. Several pieces of evidence suggested that the sites where the early mRNAs accumulate and are translated and the sites of DNA synthesis are distinct structures that coexist in the cytoplasm without being obviously connected. As mentioned, our HU washout experiment demonstrated that DNA synthesis is not initiated at the mRNA sites. Furthermore, our double-transfection experiments showed the existence of two distinct structures that can accumulate viral mRNAs during the course of infection. Several pieces of evidence demonstrated that the mRNA structures that do not colocalize with the DNA sites are core-extruded early mRNAs. Similar structures that did not colocalize with cores and that incorporated either BrUTP or the H5R antisense oligonucleotide were formed in the presence of HU and cycloheximide, conditions that block DNA replication but not early transcription (15). When BrUTP or the H5R oligonucleotide was transfected immediately after infection, the mRNA structures efficiently incorporated either of the two probes before DNA replication was initiated.

It seems most plausible to assume that transcription detected at the sites of DNA synthesis corresponds to the production of intermediate and late mRNAs, since DNA replication has been shown to induce a switch from early to late transcription (reviewed in references 21 and 22). Use of the H5R antisense oligonucleotide did not allow us to discriminate between these two processes, since this mRNA may be transcribed both early and late in infection (17). Nevertheless, our data show that the primary sites where early mRNAs accumulate (and where early proteins are most likely synthesized) and the sites of DNA replication are clearly distinct. At the molecular level their relationship is, however, clear-cut. DNA synthesis depends on the translation of viral early messengers, since a subset of these messengers encodes factors essential for DNA replication. Since our data suggested that early protein synthesis occurs in structures that are distinct from the DNA replication sites, an obvious question is how those proteins required for DNA synthesis are subsequently targeted to the replication sites. With respect to this question, the observations of Beaud and Beaud (2) are interesting. In that study it was shown that in the absence of DNA synthesis the gene product of H5R localized diffusely in the cytoplasm but that upon replication the protein was efficiently recruited to the replication sites. It is not clear at present whether this is a general behavior of VV early proteins involved in DNA synthesis, and the underlying mechanism of this targeting process remains to be investigated.

Core uncoating occurs where DNA synthesis is subsequently initiated. In agreement with our previous observations (24), we found that intracellular cores accumulated in close proximity to membranes of the ER. Our recent observation suggesting that cores may also lie on MTs (19) is not at odds with the present data. From many studies it is clear that the ER and MTs are closely interconnected structures (reviewed in reference 1). Thus, cores can be bound to MTs and also be found close to the ER. It is even conceivable that MTs aid in directing the cores toward the membranes of the ER. In our earlier study, cores that were prevented from uncoating by actD were shown to accumulate close to the ER, but in that report we had no explanation for this observation (24). The present study provided a plausible reason why cores are found next to the ER; upon their uncoating, the parental DNA appeared to associate preferentially with the cytosolic sides of ER membranes. We have tried to confirm these results biochemically, by using flotation gradients to test whether the metabolically labeled parental DNA associated with membranes upon core uncoating. The results were, however, not interpretable, most likely because the gradients were contaminated with intact virions that had remained attached to the plasma membrane after infection.

Our data fit entirely with our recent observations showing that VV DNA replication occurs in close association with the ER (29). In that study, we showed by EM that at the earliest time point tested, newly synthesized DNA appeared to occur close to the cytosolic sides of ER cisternae. Subsequently, about 45 min later in infection, these cisternae appeared to enclose the entire DNA replication site, giving the impression of cytoplasmic nuclei (29). Our present data now extend those results by showing that the parental DNA, upon leaving the core, also associated with the ER. Thus, while we have previously interpreted our EM images as suggesting that newly synthesized viral DNA perhaps actively recruited ER cisternae, our present data suggest that "old" DNA may associate with the ER even before replication has started. A logical scenario is thus that DNA replication is initiated on the cytosolic sides of the same membranes to which the parental DNA is deliv-

FIG. 9. Cores as well as the parental DNA lie close to ER membranes. All images are from cells infected for 1 h without inhibitor and labeled with anti-DNA. (A) A heavily labeled core (large arrowhead) that has apparently not yet uncoated its DNA can be seen close to the ER (small arrowhead). (B) A core (large arrowhead) weakly labeled with anti-DNA is found close to membranes of the ER (small arrowheads) that appear labeled (small arrows) on the cytosolic sides of the membranes. In this image the ER seems to bend around the DNA-containing structures. (C) Two cores can be seen (large arrowheads),of which one is unlabeled and one is heavily labeled. ER membranes are found close by (small arrowheads) and are labeled on their cytosolic sides (small arrows). In this image also, the ER membranes give the impression of bending around the DNA. Bars, 200 nm.

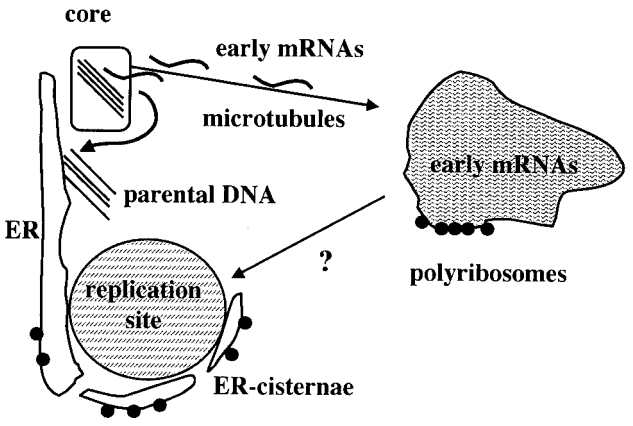

FIG. 10. Model representing our view of the cytoplasmic organization of the early stages of VV infection. After entry, intracellular cores may associate with both MTs and the ER. From these cores early mRNAs are transcribed and extruded; they then organize in an MT-dependent fashion into typical (1-μm) granular structures. The latter are located some distance from the intracellular core and recruit polyribosomes as well as other components of the host cell translation machinery, making it likely that early proteins are synthesized at this site (19). Protein synthesis early in infection is required to induce the release of the parental DNA from the core. Upon release the parental DNA may associate with membranes of the ER. Early proteins involved in DNA synthesis are somehow recruited to the parental DNA (arrow with question mark) to initiate replication on the cytosolic side of the ER. As replication proceeds, new ER cisternae are recruited to the replication sites; these will eventually form an almost completely sealed ER envelope around the site (29). As the ER wrapping proceeds, membrane-bound ribosomes will preferentially be targeted to the outer ER membrane (black dots on the ER) (see reference 29).

ered after uncoating. As replication proceeds, more cisternal ER membranes may then be recruited, such that the site is subsequently almost completely ER enclosed. Our data imply that DNA synthesis is initiated where the core delivers its parental DNA into the cytoplasm. We have attempted to address this possibility more directly by double labeling of infected cells with DAPI (to label the sites of DNA synthesis) and the anti-core antibody. We found, however, that DNA synthesis was always preceded by core degradation, making it impossible to visualize both structures at the same time.

Concluding remarks. Figure 10 summarizes our view on the cytoplasmic organization of the early stages of VV morphogenesis, which we base on the present as well as previous data. When the core is delivered into the cytoplasm, it produces a collection of early mRNAs that are extruded from the core and that organize in an MT-dependent manner at sites that locate some distance away from the cores (this study and reference 19). Upon uncoating, the core delivers the parental DNA to the cytosolic sides of ER membranes, where DNA synthesis is subsequently initiated in close association with these membranes. The DNA replication sites then develop into structures that are distinct from those where early mRNA accumulate. Since early mRNAs appear to be transported away from intracellular cores and since DNA replication may be initiated next to these cores, it follows that mRNA and DNA sites are located at distinct cytoplasmic locations. Finally, the fact that the parental DNA (this study), the newly synthesized DNA (29), and the DNA that is subsequently inserted into the newly formed virus particle (8, 9) associate with membranes of the ER suggests that these membranes harbor cellular and/or virally encoded proteins that can mediate such interactions.

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