Type I Topoisomerase Activity after Infection of Enucleated, Synchronized Mouse L Cells by Vaccinia Virus

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The time course of appearance of type I topoisomerase activity after the infection of mouse L cytoplasts by vaccinia virus was determined. When the enucleation procedure was carried out with unsynchronized cell cultures, a high level of host-cell-specific type I topoisomerase activity was found associated with the resulting cytoplasts. If cells were first synchronized by the two-cycle thymidine block method and then enucleated after release, the level of host type I topoisomerase activity was also high for S-phase-enucleated cells but was very low for cytoplasts prepared from cells previously synchronized and enucleated during either the G1 or the G2 phase. After the infection of G1-phase-enucleated cytoplasts with vaccinia virus, newly synthesized type I topoisomerase activity first appeared at about 3 h postinfection. Virosomes were isolated from the infected, synchronized cytoplasts and assayed for the presence of type I topoisomerase activity. The activity remained at the top of a sucrose gradient, well resolved from the virosome fraction, at the low salt levels (0.01 M KCl, 0.01 M Tris hydrochloride, pH 8.0) normally used in the course of virosome purification. If the sedimentation was at the higher salt concentration (0.15 M KCl) at which the enzyme shows optimal activity, type I topoisomerase cosedimented with the virosome fraction onto the sucrose gradient cushion. These results show that the type I topoisomerase activity dependent upon vaccinia virus infection may be detected with high sensitivity in G1-phase-enucleated cytoplasts. The association with virosomes is consistent with an involvement of topoisomerase activity either in DNA replication or in late transcription.

Vaccinia virus, a member of the orthopoxvirus family, contains a linear duplex DNA of 120×10^6 to 130×10^6 daltons (8, 9). Infection of susceptible cells by vaccinia virus shuts off host DNA synthesis promptly, followed by rapid inhibition of both transcription and translation of host genes. Immediately after infection, the envelope of the virus is removed by an active process. The resulting core particles contain a set of virus-specific enzymes thought to be involved in early transcription and modification of transcripts. It does not appear that any host enzymes are packaged in the course of vaccinia morphogenesis. Several comprehensive reviews have recently appeared (9, 13, 24).

We have reported previously that vaccinia cores contain a DNA-dependent type I topoisomerase (topo I) activity (1). This enzyme has been postulated to be virus specific in that it can be specifically labeled with [³⁵S]methionine after infection; its conditions for optimal activity differ significantly from those of host topoisomerases; and its molecular size on sodium dodecyl sulfate gels, 35,000 daltons, is markedly lower than that of the known host topo I. Although the biological importance of the encapsidation of this enzyme has not been established, it is reasonable to suggest that it has a significant role, along with the other intravirion enzymes, in early transcription (6).

Topoisomerase activity in eucaryotic cells is generally detected primarily in the nuclear fraction. The vaccinia virus infective cycle includes, however, little if any involvement of the nucleus, particularly during the early stages (15, 16). This permits the use of cytoplasts for study of a possible role of topo I in the process of virus replication within the cytoplasm. The present report describes the results of an initial investigation of the appearance of topo I activity in

cytoplasts after vaccinia virus infection and as a function of the particular phase of the cell cycle during which enucleation was performed, using synchronized cells. In a further series of experiments we describe conditions under which nascent vaccinia topo I associates strongly with DNA replication complexes (virosomes).

MATERIALS AND METHODS

Cells and virus. Mouse L cells were grown in suspension medium, Eagle minimal essential medium supplemented with 5% fetal calf serum, or on 60-mm plastic petri dishes in Eagle minimal essential medium supplemented with 5% fetal calf serum. Cells were normally plated at a subconfluent density, 2×10^5 to 3×10^5 per dish, and were allowed to grow overnight in an incubator at 37°C with 5% CO₂. Suspension cultures were grown to about 5×10^5 cells per ml in spinner flasks at 37°C before use. For synchronization, a low-density cell culture (1×10^5 to 1.5×10^5 /ml) was used initially. Vaccinia virus (strain WR) was grown in mouse L cells, and the titer of virus particles in the purified virus preparation was estimated by plaque assay as described by others (10).

Cell synchronization. Mouse L cells were synchronized by using the two-cycle thymidine block method with 5 mM thymidine (17). Briefly, 5 mM thymidine (Sigma Chemical Co., St. Louis, Mo.) was added to the cells at a density of 1×10^5 to 1.5×10^5 /ml, and incubation continued at 37°C for 24 h. Cells were then grown in thymidine-free medium for 7 h, after which 5 mM thymidine was again added and incubation was continued for 16 to 20 h to bring all cells to the boundary between the G1 and S phases. After removal of thymidine, cells were isolated after 2 h of release (in the S phase), after 8 h (into the G2 phase), or after 14 h (into the G1 phase) (20).

Enucleation. Cell monolayers on 60-mm petri dishes were enucleated by treatment with the drug cytochalasin B (3), using procedures described in detail by others (7). Our

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FIG. 1. Assay for topo I activity in cytoplasts obtained by enucleation of an unsynchronized culture of mouse L cells. Cell suspension (100 ml) was enucleated by the Ficoll gradient method (described in Materials and Methods) and harvested by centrifuging for 5 min in an IEC centrifuge at 1,500 rpm. Lanes: a, cells were washed free of medium and suspended in 5 ml of PBS buffer, and 10 μ l of the suspension was assayed for topoisomerase; b, cells were harvested, placed in breakage medium, and assayed; c, assay after Dounce homogenization in the same buffer; d, homogenate was centrifuged at 1,500 rpm for 5 min, and supernatant was assayed; e, pellet was resuspended in the same volume of buffer and assayed; f, control pSM1 DNA.

procedures are described in summary form. Subconfluent monolayers of cells grown in Eagle minimal essential medium plus 5% fetal calf serum were treated with 10 μ g of cytochalasin B (Aldrich Chemical Co., Inc., Milwaukee, Wis.) per ml for 15 min at 37°C. Plates were then inverted and placed aseptically in bottles two-thirds filled with prewarmed medium plus 5% serum containing cytochalasin B and centrifuged in a Sorvall RC2-B centrifuge at 8,500 rpm for 45 min in a prewarmed GSA rotor. The cells were washed with prewarmed medium supplemented with 5% fetal calf serum and incubated in the same medium for 45 min to 1 h for recovery. For large-scale enucleation, cells in suspension were centrifuged in a Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient in the presence of cytochalasin B (12). The fraction of whole cells remaining after this procedure is typically 0.2% (12), and the fidelity of enucleation was monitored by visual observation of Giemsa-stained cultures with a Zeiss photomicroscope III. In addition, we found that the homogenization conditions which resulted in efficient breakage of enucleated cells resulted in little if any breakage of any contaminating intact cells, which were then removed by differential centrifugation.

Infection of enucleated cells and virosome preparation. After the recovery period, cell monolayers were washed with serum-free medium. Virus was diluted to the required concentration in standard phosphate-buffered saline (PBS) containing 1 mM MgCl₂. A 0.5-ml sample of this virus suspension was used per plate for infection. The plates were incubated at room temperature for 30 min with intermittent rocking for uniform spreading of the virus. The virus inoculum was removed, medium plus serum was added, and incubation was continued at 37°C in a 5% CO₂ incubator. For pulse-labeling of DNA, [³H]thymidine (10 μ Ci/ml; New England Nuclear Corp., Boston, Mass.) was added at the times indicated, and after a 15-min pulse, the cells were frozen to stop further synthesis. The cells were then thawed,

the medium was removed, and cell layers were loosened in PBS by scraping with a rubber policeman. The whole scraped suspension was removed, precipitated with trichloroacetic acid (TCA), collected on filters, and counted. For virosome preparation, the cytoplasts thus obtained were broken by repeated passage with a P1000 Gilson Pipetman and centrifuged through a 25 to 40% sucrose gradient onto an 80% cushion in an SW28.1 rotor for 20 min at 20,000 rpm. Fractions were collected and assayed for enzyme activity and TCA-precipitable counts.

Enzyme assay. For the determination of enzyme activity, scraped cytoplasmic suspensions in PBS were first pelleted by centrifugation for 5 min with a Damon centrifuge at 580 $\times$ g. The supernatant was then removed, and the pellet was suspended in 100 μ l of breakage buffer (10 mM Tris hydrochloride [pH 8.0], 50 mM KCl, 5 mM EDTA) per approximately 10⁶ cytoplasts. Cytoplasts were broken by shearing through 50 to 60 cycles in a P200 or P1000 Gilson Micropipetman, and a volume of 5 to 15 μ l was used in the reaction mixtures. The topoisomerase reaction mixtures contained 20 mM Tris hydrochloride (pH 7.5), 10 mM EDTA, 30 μ g of bovine serum albumin per ml, and 0.15 M KCl in a total volume of 30 μ l. To start the reaction, 5 to 15 μ l of sample was added, the total volume of 30 μ l was adjusted with water, and the reaction mixture was incubated for 30 min at 37°C. Reactions were stopped by the addition of 5 μ l of stop mixture (4% sodium dodecyl sulfate, 25% glycerol, 60 mM EDTA, 0.1% bromphenol blue). The entire reaction mixture was loaded onto a 0.8% agarose gel and electrophoresed in E buffer (40 mM Tris hydrochloride [pH 8.0], 5 mM sodium acetate, 1 mM disodium EDTA) at constant voltage. The gels were stained with 1 μ g of ethidium bromide per ml and visualized with a transilluminator.

RESULTS

Topo I activity in mouse L-cell cytoplasts. In an initial series of experiments, we investigated the levels of topo I activity in uninfected host (mouse L-cell) cytoplasts. Figure 1 shows the results of assays for topo I activity in cytoplasts obtained by enucleation of an unsynchronized suspension culture. Activity was determined by the extent of conversion of plasmid pSM1 DNA I from the supercoiled, covalently closed form (lower band) to a series of partially relaxed forms (intermediates) in an *in vitro* assay system. Lanes a and b show that the unbroken or unhomogenized cytoplasts when suspended in PBS buffer or low-ionic-strength breakage buffer exhibited no appreciable topoisomerase activity. After Dounce homogenization, however, the sample showed appreciable topoisomerase activity (lane c). The supernatant from the homogenate (lane d) showed a consistently high level of activity, and the activity associated with the pellet fractions (lane e) was relatively less. This result thus shows that an unsynchronized cell population contains considerable topoisomerase activity in the cytoplasmic fraction, probably mostly in soluble form. We found, however, that the level of topoisomerase activity in cytoplasm preparations varies from low or none to very high levels (data not shown). This variability led us to investigate the dependence upon the cell cycle of topo I activity in cytoplasts prepared from synchronized cultures of mouse L cells.

Variation of topo I activity with cell cycle. Figure 2 shows the results of assays of cytoplasmic activity in different phases of a synchronized cell suspension. Mouse L cells previously synchronized by the thymidine block method (see Materials and Methods) were enucleated in different phases of the cell cycle, and the activity was assayed after homog-

enization of the cytoplasts in breakage medium. The results demonstrated a clear variation of topo I activity with the cell cycle. In particular, topoisomerase activity was especially prominent in the S phase, whereas there was little or no observable activity in either the G1 or the G2 phase.

DNA synthesis in virus-infected, G1-synchronized cytoplasts. Experiments employing autoradiography have shown that virus replication complexes (virosomes) are located exclusively in the cytoplasm and that DNA synthesis occurs in vaccinia-infected enucleated mouse L cells (16), although production of mature progeny virus is blocked in these circumstances (15). In the present investigation, we examined vaccinia virus-specific DNA synthesis in cells enucleated in the G1 phase. Figure 3 shows the results of [^3H]thymidine pulse-labeling in vaccinia-infected cytoplasts prepared in the G1 phase of a synchronized culture. The data show the rate of incorporation of label as a function of time postinfection. The incorporation of [^3H]thymidine was maximal at about 3.5 h postinfection and thereafter dropped off rapidly. In a control experiment with uninfected cytoplasts, little incorporation of thymidine was observed, suggesting that the incorporation of [^3H]thymidine in the infected cell cytoplasts is due to virus-specific DNA synthesis.

Appearance of topo I activity in virus-infected, G1-synchronized cytoplasts. To investigate the relationship between virus-specific DNA synthesis and topoisomerase activity we performed *in vitro* assay experiments with infected cytoplasts at various times after infection. Figure 4 shows the kinetic pattern of topoisomerase activity as a function of time postinfection. Experiments employing cytoplasts enucleated in the G1 phase of the cell cycle indicated that activity appears abruptly at 3 h postinfection and is maintained until at least 5 to 6 h postinfection. Similar experiments with enucleated unsynchronized cell cultures also showed an increase level of topo I activity over the background cell cytoplasmic activity, at about 3.5 to 4 h postin-

FIG. 2. Assay for topo I activity in preparations of cytoplasts obtained by enucleation of synchronized cells in suspension. The procedures for synchronization and for enzyme assay are described in Materials and Methods. In each phase as indicated, the cell culture was withdrawn and enucleated as described in the legend to Fig. 1 and was then assayed. Lanes: a, S phase; b, G2 phase; c, G1 phase.

FIG. 3. Vaccinia virus DNA synthesis in enucleated mouse L cells. Subconfluent monolayers of G1-phase cells were enucleated in plates as described in Materials and Methods and infected at a multiplicity of infection of 10. At the indicated times, postinfection, plates were pulse-labeled for 15 min with 10 μCi of [^3H]thymidine per ml, and TCA-precipitable counts were determined. Symbols: $\bullet$, uninfected cytoplasts; $\circ$, infected cytoplasts.

fection (unpublished data). It should be noted that the time of occurrence of the maximum rate of viral DNA synthesis (Fig. 3) is about the same as that for the first appearance of topoisomerase activity in cytoplasts. This raises the possibility that an appreciable level of topoisomerase activity is needed for replication to occur with high efficiency, perhaps by relieving torsional stress at the replication fork. The levels of input vaccinia virus in these experiments were calculated to be far too low to produce detectable levels of input topoisomerase activity under the assay conditions.

Association of topo I activity with virosomes. Virosomes were labeled during the period of 2.5 to 3.75 h after infection of cells enucleated during the G1 phase of the cell cycle and

FIG. 4. Time course of the appearance of topoisomerase activity after infection of synchronized, enucleated mouse L cells. Cytoplasts prepared from cells which had been synchronized and halted in the G1 phase were infected with vaccinia virus as described in Materials and Methods. At various times after infection, cytoplasts were disrupted, and 10 μl was removed for assay. The results of the assay with pSM1 DNA were determined at times after infection of 0, 1, 2, 3, 4, and 5 h and are shown in lanes a through f, respectively.

FIG. 5. Assays for topo I activity of viroosome-containing fractions from a sucrose density gradient. Approximately 200 ml of L cells at a cell density of 5×10^5 /ml was enucleated by the gradient method (see Materials and Methods), plated in monolayers in 100-mm-diameter petri dishes, and incubated for about 1 h in medium free of cytochalasin B. The plates were then washed with serum-free Eagle minimal essential medium and infected with virus at a multiplicity of infection of 10. At 3.75 h postinfection, plates were frozen at -20°C to stop further incorporation; plates were then thawed, medium was removed, and cytoplasts were harvested and suspended in 1 ml of high-salt medium (0.01 M Tris hydrochloride [pH 8.0], 0.15 M KCl, 0.005 M EDTA). This was then homogenized, loaded on a 25 to 40% sucrose gradient containing 0.15 M KCl with an 80% cushion, and centrifuged at 20,000 rpm for 20 min in an SW28.1 rotor. Fractions were collected from the bottom of the tube. Part of each fraction was used for the enzyme assays shown here, and the remainder was used to determine incorporation of radioactivity by TCA-precipitable counts (not shown). Lanes a through e represent fractions taken from the gradient, beginning with the bottom (at left). The location of the virosomes corresponded approximately to fractions b through e.

were isolated by density gradient sedimentation as described in Materials and Methods. The viroosome band lies between fractions 3 and 5 from the bottom of the gradient (as determined from TCA-precipitable counts). Control uninfected cell cytoplasts, incubated during the same period with [^3H]thymidine and then sheared and centrifuged on an identical gradient, revealed no appreciable incorporation of [^3H]thymidine. Under these low-salt conditions, topo I activity remained at the top of the gradient and showed no association with the viroosome fraction (data not shown). Thus, although topo I appears to be newly synthesized during the infectious cycle of vaccinia virus, its binding to the DNA replication complex is very weak under conditions in which virosomes are normally isolated. Previous investigations of the ionic-strength dependence of the vaccinia topoisomerase activity (1) have shown that little activity is expected in the low-ionic-strength density gradient buffer, suggesting in turn that binding of the topoisomerase to virosomes might be greatly reduced under these conditions.

To determine the association of topoisomerase activity with virosomes at salt concentrations near the optimum for enzyme activity, cytoplasts from cells synchronized in the G1 phase were prepared and used as described immediately above, except that the salt concentration during the sucrose density gradient centrifugation was adjusted to 0.15 M KCl. Figure 5 shows the results of the assay of fractions from near the bottom of the density gradient under these conditions. It is clear that at moderate salt concentrations the topoisomerase activity is strongly associated with the fractions containing virosomes. Control experiments (not shown) in which mock-infected synchronized cytoplasts were assayed under these conditions again showed no detectable

topoisomerase activity. Thus, topo I is both synthesized at a time approximately coincident with the initiation of DNA replication and, under the appropriate salt conditions, is strongly associated with virosomes.

DISCUSSION

Topoisomerases make up a class of DNA-dependent enzymes which catalyze the reversible scission and ligation of the phosphodiester-sugar backbone chain (for recent reviews, see references 22 and 23). The subclass which has been designated type I is further characterized by independence from added energy sources and by the ability to catalyze local duplex winding alterations in steps of a single turn (360°). The eucaryotic topo I's, unlike the enzyme from *Escherichia coli*, do not require Mg^{2+} or other divalent metals as cofactors. Topo I's are conveniently assayed with closed circular DNA, the linking number of which is changed in unit steps during the isomerization. No biological function has yet been assigned to any of the eucaryotic topo I's; and in yeasts topo I has been shown to be nonessential (R. Sternglantz, private communication). In *Drosophila* polytene chromosomes, however, it has recently been shown by immunofluorescence that topo I is preferentially associated with transcriptionally active regions (5). Since the vaccinia enzyme is primarily located within the viral core, a role in early transcription has also been suggested for the viral topoisomerase (1), as is known to be the case with other encapsidated viral enzymes (2, 14). The vaccinia enzyme, with a molecular size of about 35,000 daltons (1, 6), is the smallest reported topo I, and it is the first topoisomerase to have been found packaged in an animal virus.

We report here the results of an investigation of the early stages of the appearance of topo I activity after infection of mouse L cells by vaccinia virus (WR strain). In addition, we describe conditions under which the newly synthesized vaccinia virus enzyme activity can be detected without interference from the host-cell topo I. We first investigated the presence of topo I activity in cytoplasts prepared from mouse L cells. We found that the levels of cellular topoisomerase activity in such cytoplasts are subject to marked variation depending upon the phase of the cell cycle during which enucleation takes place. Thus, topo I activity is readily evident in cytoplasts enucleated in the S phase but is nearly absent from cytoplasts enucleated in either the G1 or the G2 phase.

Previous reports of the cell-cycle dependence of the appearance of topo I activity, using intact mammalian cells, gave rise to conflicting conclusions. Rosenberg et al. (19), using a normal human lymphocytic cell line, detected high levels of topo I activity in cell extracts prepared during the S phase and very little if any topoisomerase activity in cell extracts prepared in the G1 or G2 phase, in substantial agreement with our results. Champoux and co-workers (4), on the other hand, were unable to detect any dependence of the level of topo I activity upon the cell cycle in mouse 3T3 cells. The origins of this discrepancy are not known, but the use of quite different cell lines makes direct comparisons tentative. Since both earlier reports dealt with whole cells, the observations in each case refer primarily to enzyme activity associated with the nuclear fraction.

For the enucleated cell preparations used in the present study, it seems likely that we are observing newly synthesized cellular topo I in transit to the nucleus. The apparent absence of topo I activity from cytoplasts prepared from quiescent cells can be contrasted to results obtained with fractionated whole CHEF/18 Chinese hamster embryo fibro-

blast cells (18). In this case, a group of six enzymatic activities involved in DNA replication was present mainly in the cytoplasmic fraction in quiescent cells. No evidence has, however, been presented that topo I is associated with DNA replication in eucaryotic cells, although an apparent association with transcriptionally active chromatin has been reported (5). In any event, for the study of the vaccinia-dependent activity, the use of G1- or G2-synchronized cytoplasts having negligible background is essential.

The use of enucleated cells to support infection by vaccinia virus was first demonstrated by Prescott et al. (16), although later studies showed that the course of infections proceeds only through the replication of DNA and the expression of late genes (10, 11). The syntheses of viral DNA and of both early and late polypeptides do, however, proceed with normal timing in cells that have been enucleated with cytochalasin B (15). More recent evidence indicates that the block to the production of progeny virus probably does not indicate an absolute requirement for participation of the host nucleus, since a mutant vaccinia strain has been obtained which is able to replicate fully in enucleated cells (21). These considerations support the use of enucleated cell cultures for the study of the appearance of virus-specific enzyme activities after infection. Experiments are in progress to ascertain the relationship of vaccinia-specific topo I synthesis to late functions in the virus life cycle.

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