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Association between the p170 Form of Human Topoisomerase II and Progeny Viral DNA in Cells Infected with Herpes Simplex Virus Type 1

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Endogenous host topoisomerase II acts upon herpes simplex virus type 1 (HSV-1) DNA in infected cells (S. N. Ebert, S. S. Shtrom, and M. T. Muller, J. Virol. 56:4059–4066, 1990), and cleavage is directed exclusively at progeny viral DNA while parental DNA is resistant. To evaluate the possibility that HSV-1 induces topoisomerase II activity which could account for the preferential cleavage of progeny viral DNA, we assessed topoisomerase II cleavage activity on cellular and viral DNA substrates before and after the initiation of viral DNA replication. We show that cleavage of a host gene in mock-infected cells was similar to that observed in HSV-1-infected cells, regardless of whether viral DNA replication had occurred. In addition, quantitative measurements revealed comparable amounts of topoisomerase II activity in infected and mock-infected cells; thus, HSV-1 neither induces nor encodes its own type II topoisomerase and cleavages in vivo are due to a preexisting host topoisomerase. Human cells contain two isozymes of topoisomerase II (p170 and p180), encoded by separate genes. Through the use of isozyme-specific antibodies, we demonstrate that only p170 was found to be cross-linked to HSV-1 DNA even though both forms were present at nearly constant levels in HSV-1-infected cells. Immunofluorescence revealed that by 6 h postinfection, p170 becomes redistributed and localized to sites of active viral DNA synthesis. The data suggest that p170 gains preferential access to replicated viral DNA molecules, which explains why topoisomerase II activity is concentrated on progeny DNA.

Eukaryotic type II DNA topoisomerases (EC 5.99.1.3) make transient double-strand breaks in DNA (for reviews, see references 40–42). Topoisomerase II is also a major component of the nuclear matrix (4, 13) and may be involved in chromosome condensation and structure (1, 38, 44). Because topoisomerases affect the integrity of DNA, they have been implicated in other central nuclear events including transcription (5, 24), replication (5, 11, 15, 45), and recombination (2, 9).

At least two forms of topoisomerase II exist in human cells, a 170-kDa (p170) form and a 180-kDa (p180) form (12, 37). The two forms are genetically distinct (8, 37) and are subject to independent regulation. For example, changes in intracellular concentrations of p170 are tightly linked to the cell cycle. Peak concentrations of p170 are achieved in G_2-M phase of mitosis and decline thereafter to minimal levels following mitosis (18). In contrast, p180 is maintained at a relatively constant intracellular concentration throughout the cell cycle (43).

Genetic studies in yeasts demonstrated that topoisomerase II is essential for the completion of mitosis (19, 39). Newly replicated daughter chromosomes are intertwined and cannot be resolved in the absence of functional topoisomerase II activity (38, 39). Because of this critical role in the cell cycle and because of its potential ability to behave as a DNA cleavage (and damaging) agent, topoisomerase II has served as the target for several antineoplastic compounds (23, 29). One such compound, VM26, which selectively interferes with the religation step of the topoisomerase II-DNA reaction (6), is useful for mapping the locations of topoisomerase-mediated DNA cleavages (15, 27, 44).

Analysis of a large number of sequences cleaved by topoisomerase II in vitro led to the derivation of a degenerate consensus sequence (RNYNNCNNGY/NGKTNYNY, where R = purine, Y = pyrimidine, K = G or T, N = any base, and cleavage occurs at Y/N) (34, 35). We previously demonstrated that the herpes simplex virus type I (HSV-1) genome contains numerous consensus topoisomerase II cleavage sites, at least some of which are cleaved in infected cells following the initiation of HSV-1 DNA replication (15). We also showed that host topoisomerase II, and not a viral-encoded enzyme, was responsible for the observed cleavages (15). As noted above, multiple forms of topoisomerase II exist within mammalian cells, and although host topoisomerase II has been implicated in the replicative process for several viruses in addition to HSV-1 (3, 22, 32, 45, 46), there is no indication which topoisomerase II isozyme may be responsible for these observations.

In the present study, we show that topoisomerase II activity persists in HSV-1-infected cells and that both forms are present throughout the infection; however, only p170 topoisomerase II was found to be cross-linked to HSV-1 DNA in infected cells even though both p170 and p180 could be similarly cross-linked to DNA in vitro. These results suggested that p170 is the primary agent which induces double-strand DNA cleavages on progeny viral DNA molecules. Consistent with this hypothesis, we show that p170 is redistributed to sites of active viral DNA replication in infected cells.

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MATERIALS AND METHODS

Cells and virus. HeLa and LU3-7 cells were cultured in Dulbecco's modified Eagle medium supplemented with 5% defined, iron-supplemented bovine calf serum (HyClone Laboratories, Logan, Utah) and gentamicin sulfate (50 μg/ml). HeLa cells were also maintained as predominantly single-cell suspension cultures in spinner flasks with RPMI 1640 medium supplemented with 7.5% defined, iron-supplemented bovine calf serum (HyClone) and gentamicin sulfate (50 μg/ml). LU3-7 cells were provided by L. Johnson (10). HSV-1 (KOS strain) was propagated as described previously (15). All infections were performed at an input multiplicity of 25 PFU per cell.

Decatenation assays for topoisomerase II activity. Topoisomerase II activity was measured by using a kinetoplast DNA (KDNA) as described previously (28). KDNA, the mitochondrial DNA of *Crithidia fasciculata*, is a catenated network of topologically interlocked DNA rings, the majority of which are 2.5-kb (31) monomers. Type II topoisomerases, but not topoisomerase I or nucleases, have the ability to decatenate KDNA and generate the monomer DNA that is covalently closed circular DNA (for a review, see reference 25); therefore, decatenation is a highly specific assay for topoisomerase II. Furthermore, since KDNA networks are large (relative to the monomers) separation is achieved after only a few minutes of electrophoresis. Gels are run in the presence of an intercalator (ethidium bromide) to resolve various DNA forms (linear, covalently closed, and nicked circular DNA monomers). Reaction mixtures contained 0.2 μg of KDNA (final volume, 30 μl), 50 mM Tris-HCl (pH 8), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM each dithiothreitol and ATP, and 30 μg of bovine serum albumin (BSA) per ml (topoisomerase II reaction buffer). The reaction mixtures were incubated for 15 min at 37°C, and the reactions were terminated with 0.1 volume of stop buffer (5% Sarkosyl, 0.025% bromophenol blue, 50% glycerol).

Glycerol gradient fractionation of topoisomerase II. Crude extracts were prepared by treating the cells with 0.8 M NaCl–20 mM 2-mercaptoethanol in TEMP buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 4 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride) followed by centrifugation at 100,000 × *g* for 1 h. The supernatant was loaded onto a 15 to 40% glycerol (in TEMP buffer) gradient and centrifuged in an SW41 rotor at 35,000 rpm for 65 h. Fractions were collected from the bottom of the gradient and assayed for topoisomerase II activity by decatenation of KDNA (as described above). As an internal marker, topoisomerase I (100 kDa) was assayed by relaxation of supercoiled plasmid DNA in buffer without Mg^{2+} or ATP, as described previously (36). To reduce the total number of assays, fractions were pooled in pairs (fractions 1 and 2 were mixed together, prior to assay, and so on) and topoisomerase II activity measurements were performed. The relative topoisomerase II activity was determined by densitometry of the decatenation products resolved on an agarose gel. Peak topoisomerase II fractions were assigned a relative value of 100. The marker (topoisomerase I) corresponded to the fraction that relaxed the majority of the supercoiled DNA substrate.

Detecting and mapping topoisomerase II cleavages in vivo. Cleavages were detected as previously described (15), with the following modifications. For detection of cleavages in LU3-7 cells, the medium was removed, the cells were lysed by the addition of 1% sodium dodecyl sulfate (SDS), and the DNA was then purified by standard methods as described previously (15). For mapping topoisomerase II cleavages in the mouse thymidylate synthase gene, LU3-7 DNA was digested with *Hin*dIII and subjected to agarose gel electrophoresis followed by Southern blot transfer to GeneScreen Plus (DuPont) membrane. This membrane was subsequently hybridized with a probe derived from the 5′ end of the second intron of the mouse thymidylate synthase gene (10). This probe was made by random-prime labelling (Amersham Corp., Arlington Heights, Ill.) of a *Hin*dIII-*Sst*I fragment from plasmid pH3.6kb (provided by K. Jolliff) (21) with [α-^{32}P]dATP (ICN, Irvine, Calif.). For detection of topoisomerase II cleavages in HSV-1 DNA, Southern blots were probed with the 321-bp *Kpn*I-*Xho*I fragment derived from the immediate-early gene 1 (IE-1; encodes ICP0) (plasmid p111) as previously described (15).

Trapping topoisomerase II-DNA cleavage intermediates in vitro. HeLa topoisomerase II activities were partially purified (Biorex-70 column) and contained a mixture of p170 and p180. The enzyme was incubated with 4 μg of pRYG DNA, which contains clustered topoisomerase II recognition elements (33), in 80 μl (final volume) of cleavage buffer (30 mM Tris-HCl [pH 7.6], 60 mM KCl, 8 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 3 mM ATP, and 30 μg of BSA per ml) for 30 min at 30°C in the presence of increasing concentrations of VM26 (0, 50, 100, and 200 μM). Reactions were terminated by addition of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, divided into equal aliquots and loaded onto a 7% polyacrylamide gel. The topoisomerase II forms were detected by immunoblotting (see below) with isozyme-specific antibody probes.

Trapping topoisomerase II-DNA cleavage intermediates in vivo. Mock- or HSV-1-infected HeLa cells (at 12 h postinfection [12 hpi]) were left untreated or were treated with VM26 (100 μM) for 30 min in serum-free Dulbecco's modified Eagle medium (1 ml per 100-mm-diameter dish). The medium was then removed, and cells were harvested by direct lysis of 1% Sarkosyl (3 ml per dish). Equivalent volumes of lysate were loaded onto a CsCl step gradient (27) and centrifuged at 31,000 rpm for 18 h at 20°C with an SW41 rotor. These gradients separate macromolecules on the basis of density differences such that DNA bands at the lowest step (nominal density, 1.7 g/cm^3) and protein bands at the top step (nominal density, 1.29 g/cm^3). Fractions (200 μl each) were collected from the bottom, and 50 μl was mixed with 100 μl of 25 mM sodium phosphate buffer, pH 6.5, and applied to Hybond ECL membrane (Amersham) in a slot blot manifold. Blots were then processed for immunoblot analysis with the different topoisomerase II antibodies as described below.

Hybrid selection of covalent topoisomerase II–HSV-1 DNA complexes. An HSV-1-specific DNA (*Nco*I-*Sal*I fragment of IE-1) and a control DNA (salmon sperm; Sigma Chemical Co.) were denatured and applied to Hybond C-extra membrane in a slot blot manifold. The membranes were prehybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.25% nonfat dry milk at 65°C overnight. DNA-containing fractions from the CsCl step gradients (described above) were pooled, denatured with 3 N NaOH for 5 min, neutralized with 3 N HCl and 1 M Tris-HCl (pH 7.5), and used for hybridization of the membranes containing the HSV-1 or salmon sperm DNA. Following overnight hybridization at 65°C, the filters were washed in 1× SSC for 30 min at room temperature and in 1× SSC and 0.1% SDS for 2 h at 65°C and briefly rinsed with 0.1× SSC at room temperature. Topoisomerase II was detected by immunoblotting analysis.

Immunoblot analysis. Nuclear extracts from mock- or HSV-1-infected HeLa cells (multiplicity of infection = 25 PFU per cell) were prepared as follows. At 12 hpi, the cells were washed twice with cold TD buffer (100 mM NaCl, 20 mM KCl, 0.5 mM Na_2HPO_4, and 20 mM Tris-HCl [pH 7.45]) and scraped from

100-mm petri dishes into 2 ml of TD buffer per dish. The cells were then concentrated by low-speed centrifugation (1,500 rpm, 5 min), resuspended in 2.5 ml of cold buffer A (100 mM NaCl, 50 mM KCl, 20 mM Tris [pH 7.5], 0.1% Triton X-100), and incubated on ice for 5 min before another low-speed centrifugation to pellet nuclei (2,000 rpm, 10 min). The nuclei were lysed by direct addition of SDS (to 1% [vol/vol]) followed by brief sonication. Protein concentrations were determined by using Micro BCA Protein Assay Reagent (Pierce, Rockford, Ill.) according to the manufacturer's specifications. Proteins were separated by standard SDS-PAGE (the resolving gel contained 7% acrylamide). Following separation, proteins were transferred to Hybond C-extra membrane (Amersham) overnight (4°C) at a constant current of 200 mA in 190 mM glycine and 30 mM Tris base. The membranes were briefly rinsed in water and equilibrated for 15 min in TBST buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.05% Tween 20), before incubation in TBST containing 5% nonfat dried milk (BLOTTO) and 0.01% sodium azide. Affinity-purified primary antibodies specific for either the p170 or the p180 form of topoisomerase II (provided by F. H. Drake) (8) were diluted 1:1,000 in TBST and incubated with the membranes for 6 h at room temperature. The membranes were washed three times with TBST for 5 min each and incubated for 1 h with ^{125}I-protein A (0.5 μCi/ml in TBST). The membranes were again washed three times (5 min each) with TBST, air dried, and exposed to film. For some applications, we used enhanced chemiluminescence (Amersham) for visualization of the immunoblot signals. Similar results were obtained with both detection systems.

Indirect immunofluorescence. This method was adapted from that described by Earnshaw et al. (14) as follows. HeLa cells were seeded on coverslips in 100-mm-diameter dishes. Solution volumes indicated refer to those used per 100-mm petri dish. At the indicated times postinfection, the cells were washed twice (5 min for each dish) with 6 ml of D-PBS (8.06 mM $Na_2HPO_4 \cdot 7H_2O$, 1.47 mM KH_2PO_4 [pH 7.1], 137 mM NaCl, 0.27 mM KCl, 0.68 mM $CaCl_2$, and 0.49 mM $MgCl_2$). The cells were then fixed for 10 min at room temperature with 3.7% formaldehyde in D-PBS and washed twice with 4 ml of D-PBS (5 min each) and once with 0.1% SDS and 1% 2-mercaptoethanol in D-PBS for 10 min at room temperature. The cells were then rinsed briefly in D-PBS and washed twice (5 min each) with 4 ml of KB buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, and 0.1% BSA). The cells were permeabilized with 3 ml of −20°C methanol for 2 min and then washed twice again with 4 ml of KB for 5 min each. Primary antibody dilutions were prepared in KB buffer. The 6898 mouse monoclonal antibody (a gift from H. S. Marsden), which is specific for the HSV-1 65-kDa DNA-binding protein (DBP) (16, 17), was diluted 1:1,000, while the affinity-purified p170 topoisomerase II rabbit polyclonal antibody was diluted 1:10. The primary antibody solutions were added to the coverslips containing HeLa cells as follows. A coverslip was removed from the last KB wash, excess buffer was carefully removed by blotting the edge of the coverslip with a tissue, and 20 μl of primary antibody solution was added to the cell side of the coverslip, which was placed cell side down in a 35-mm petri dish and placed in a 37°C humidified incubator (95% air, 5% CO_2) for 3 h. Following the primary antibody incubations, the cells were twice washed in the 35-mm petri dish with 1 ml of KB buffer for 10 min each while the coverslips were turned cell side up during the first wash. All subsequent manipulations were performed in the dark. Secondary antibodies (Cappel Laboratories, West Chester, Pa.) were diluted 1:150 in D-PBS, and 35 μl was applied to each coverslip in the same manner as described for the addition of primary antibody. The coverslips were then left for 1 h at 37°C in a humidified incubator (95% air, 5% CO_2). Upon completion of incubation with secondary antibodies, the coverslips were washed as they were after the primary antibody incubation and mounted onto slides with 50% glycerol in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. The secondary antibody used for p170 visualization was a goat anti-rabbit antibody conjugated to rhodamine isothiocyanate. The secondary antibody used to detect the 65-kDa DBP was a goat anti-mouse antibody conjugated to fluorescein isothiocyanate. Fluorescence generated from these two different secondary antibodies was discriminated through the use of exclusion filters as previously described (17). Pictures were taken with Kodak T-Max 400 film (ASA pushed to 3,200) with a 35-mm camera mounted on an Olympus BH-2 microscope.

RESULTS

Analysis of topoisomerase II in HSV-1-infected cells. Host topoisomerase II cleaves HSV-1 DNA following the initiation of viral DNA replication; parental genomes are completely protected from cleavage (15). The prevalence of cleavage activity on postreplicative HSV-1 DNA could result from an induction of enzyme activity or a preferential cleavage of replicated viral DNA (relative to cellular DNA). To test these hypotheses, we examined topoisomerase II cleavage of a host gene in mock- and HSV-1-infected cells. We previously demonstrated VM26-dependent double-strand cleavages of HSV-1 DNA in infected cells using Southern blot analysis (15), and this method was used here to detect cleavage of a host gene. The mouse thymidylate synthase gene is well characterized, and it contains topoisomerase II consensus match sites which are cleaved by purified topoisomerase II in vitro (21), thereby making it an attractive candidate target for cleavage by topoisomerase II in vivo. In addition, detection of topoisomerase II cleavages in this gene was enhanced in the LU3-7 cell line, which contains approximately 50 amplified copies of the mouse thymidylate synthase gene per cell. Double-strand cleavages were clearly seen in the thymidylate synthase gene in LU3-7 cells which had been treated with VM26 (Fig. 1A). The predominant cleavage site was located near the transcriptional initiation sites in the mouse thymidylate synthase gene. Although infection of LU3-7 cells with HSV-1 results in typical cytopathic effects and eventual cell death, dramatic alterations in the pattern and intensity of topoisomerase II cleavage in the thymidylate synthase gene in response to HSV-1 infection were not observed at either 2 or 12 hpi (Fig. 1A). Thus, infection by HSV-1 does not significantly alter topoisomerase II cleavage activity on a host gene. In contrast, VM26-dependent cleavage of HSV-1 DNA was markedly enhanced at 12 versus 2 hpi (Fig. 1B). The region of the HSV-1 genome immediately 5′ of the transcriptional initiation site for IE-1 (which encodes ICP0) contains numerous sites which match the consensus cleavage sequence for topoisomerase II, and we previously demonstrated that this region was cleaved by purified topoisomerase II in the presence of topoisomerase II poisons such as m-AMSA and VM26 (15). Furthermore, cleavage was observed in this region following VM26 treatment of infected Vero, BHK, and HeLa cells (15). The observation that this region displayed double-strand breaks in response to VM26 treatment at 12 hpi, but not 2 hpi, is consistent with previous analyses showing that topoisomerase II cleavage was not detectable on HSV-1 DNA prior to the onset of viral DNA replication at 3 hpi. Since topoisomerase II cleavage of a cellular gene was essentially unaffected by HSV-1 infection, we conclude that endogenous topoisomerase II activity is likely to

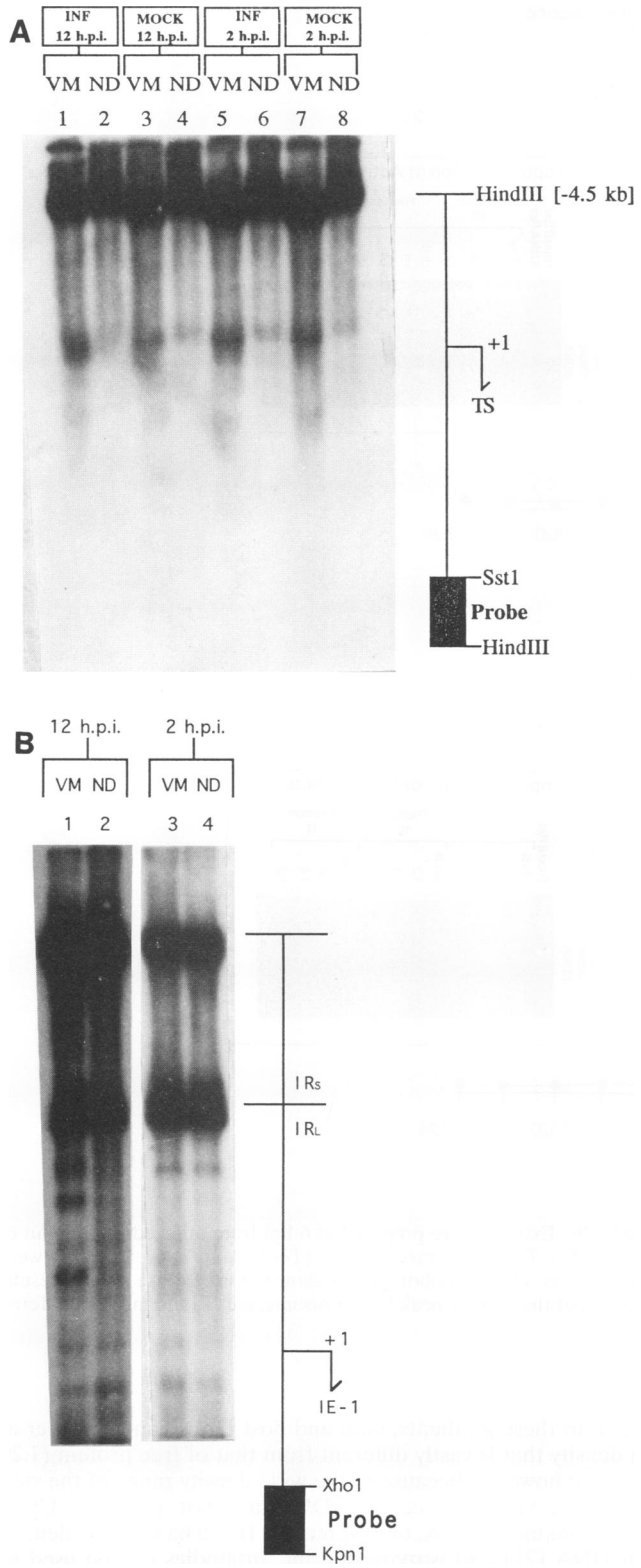

FIG. 1. Topoisomerase II cleavage of cellular and viral DNA in HSV-1-infected cells. LU3-7 cells were infected with HSV-1 at an input multiplicity of 25 PFU per cell and treated with VM26 for 30 min followed by direct lysis with SDS buffer at either 2 or 12 hpi. (A) A Southern blot of mock-infected or infected LU3-7 DNA (40 μg per lane) digested with *Hin*dIII and probed with the cellular thymidylate synthase gene by using a fragment coterminal with the *Hin*dIII cleavage site 3′ of the transcriptional initiation site (diagrammed to the right of the blot). (B) A Southern blot of mock and infected LU3-7 DNA (40 μg per lane) digested with *Kpn*I and probed with IE-1 by using a fragment coterminal with the *Kpn*I site 3′ of the transcriptional initiation site for IE-1 (diagrammed to the right of the blot). Abbreviations: VM, VM26; ND, no drug; MOCK, mock-infected LU3-7 cell DNA; INF, HSV-1-infected LU3-7 DNA; TS, mouse thymidylate synthase gene; IRS, short inverted repeat; IRL, long inverted repeat. Sites of transcriptional initiation are marked +1.

be responsible for, but not limited to, cleavage of newly replicated viral DNA.

We next measured topoisomerase II enzymatic activity in mock- and HSV-1-infected cell extracts using a decatenation assay (see Materials and Methods). Topoisomerase II can resolve interlocked kinetoplast DNA rings in the presence of $MgCl_2$-ATP, and this activity has been exploited as a means of assessing topoisomerase II enzymatic activity in vitro (26, 28). The smaller circular DNA monomers enter an agarose gel while much larger catenated DNA remains in the wells. In crude extracts, especially from infected cells, the assay was compromised by potent nucleases which caused extensive degradation of KDNA substrate. Since topoisomerase II is generally larger than most nucleases, extracts were size fractionated on glycerol gradients. An internal sizing marker for these gradients was host topoisomerase I (36) (native molecular weight, 100,000) which was assayed by relaxation of plasmid DNA in an EDTA-containing buffer to eliminate nuclease problems. The fractionation profile (Fig. 2) reveals a single peak of topoisomerase II activity in both mock-infected and infected samples. On the basis of reference to the marker, there was no difference in size between mock-infected and infected activities. Titrations of active fractions from mock-infected and infected cells reveal similar amounts of topoisomerase II activity in both at 6 hpi (Fig. 2). Preferential cleavage of HSV-1 progeny DNA does not appear to be due to induction of a topoisomerase II activity after infection. We have searched for novel activities at later times postinfection, using different biochemical fractionations (ion exchange chromatography); however, the results were uniformly negative. The total amount of topoisomerase II activity in mock- versus HSV-1-infected cells appears to be similar (as reflected in Western blotting [immunoblotting] experiments; see below). Additionally, the data are not consistent with the possibility that HSV-1 induces a novel type II topoisomerase that is biochemically distinct from that of the host.

These data suggest that HSV-1 DNA is cleaved by preexisting host topoisomerase II in infected cells. As mentioned above, human cells express p170 and p180 forms of topoisomerase II. The cleavage analyses performed thus far do not differentiate between these isozymes, since both induce double-strand breaks in DNA in vitro (see below). We utilized isozyme-specific antibodies to determine whether the p170 and/or p180 was present in HSV-1-infected cells. Mock-infected and infected HeLa cell nuclei were lysed by the direct addition of SDS buffer at 12 hpi. Equal volumes of nuclear lysates, representing equal numbers of mock-infected or infected cells, were separated by SDS-PAGE and analyzed by Western blotting, using isozyme-specific antibodies. Both isozymes are present in mock- and HSV-1-infected HeLa cells (Fig. 3). HSV-1 infection resulted in a small and somewhat variable decrease in the amount of p170 and p180 polypeptides (infected cells usually showed a 5 to 20% reduction in polypeptide on a per-cell basis); however, variability may be attribut-

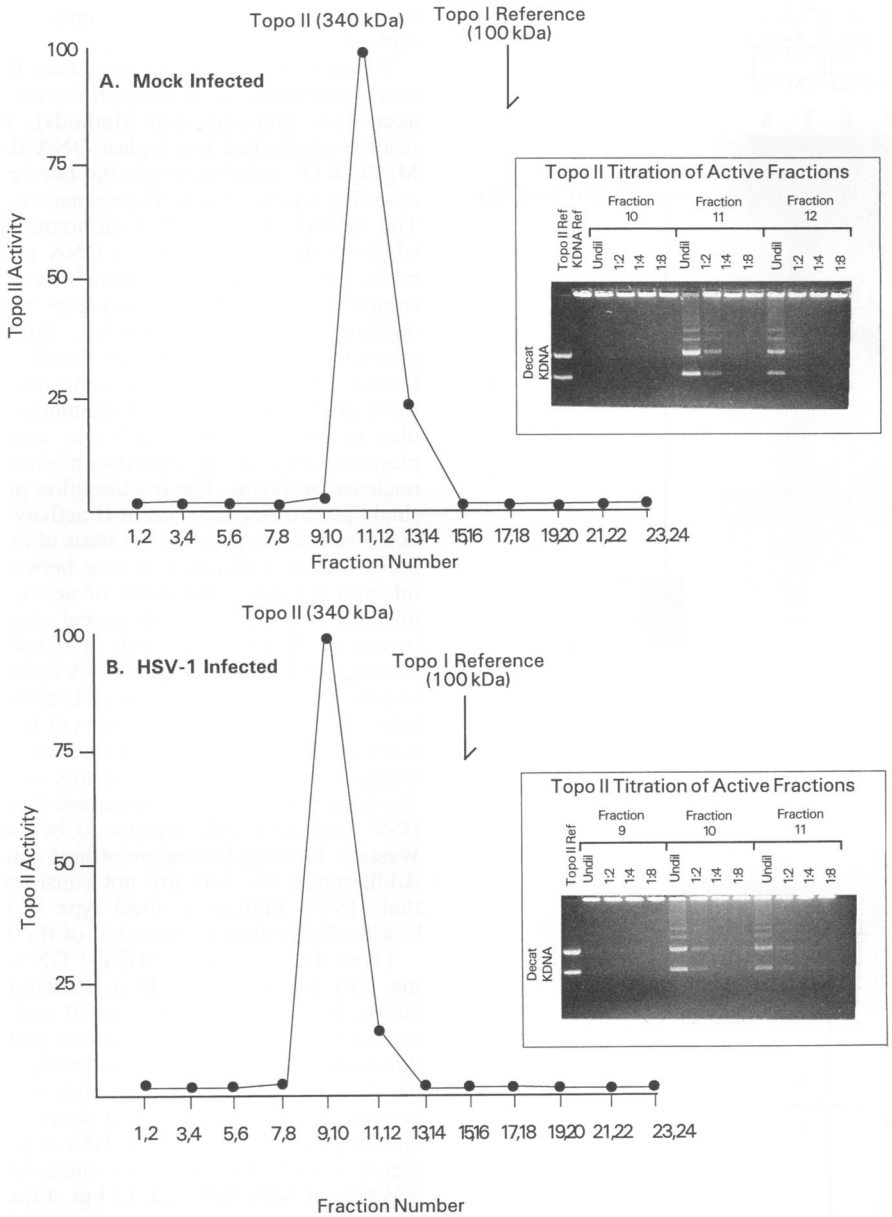

FIG. 2. Analysis of topoisomerase II activity in mock- and HSV-1-infected cells. Extracts were prepared at 6 hpi from infected cells (input of 25 PFU per cell) and from an equal number of mock-infected HeLa cells (2×10^8). Topoisomerase I and II (Topo I and Topo II) assays were performed after glycerol gradient fractionation as described in Materials and Methods. The inset photographs show topoisomerase II assay results in which the most active fractions were titrated by serial twofold dilutions. Note that the activity peak for topoisomerase II (and marker) differed by one fraction between the two gradients.

able to cell loss due to lysis associated with harvesting the virus-infected cells.

Isozyme-specific detection of VM26-induced cross-linked protein-DNA complexes. Since both forms of topoisomerase II were present in infected cells at a time when strong viral DNA cleavages occur, either or both may be responsible for the observed cleavages. We devised the following strategy to address this issue. Mock- and HSV-1-infected HeLa cells were treated with VM26 for 30 min at 12 hpi followed by detergent lysis to trap topoisomerase II in a covalent complex with DNA (27, 28). The resulting covalent protein-DNA complexes were then separated from free protein by using CsCl step gradients (27). In these gradients, viral and host DNA band together at a density that is vastly different from that of free protein (1.29 g/cm^3); however, because of the wide density range of the step gradient, viral and cellular DNA are not resolved. Upon cross-linking to DNA, topoisomerase II will band at the density of DNA (27) and isozyme-specific antibodies can be used to probe DNA- and protein-containing fractions in Western slot blots to identify which isozyme is covalently bound to DNA. As shown in Fig. 4A, only p170 was identified in the DNA peak fractions of the gradient, demonstrating that it can be readily trapped in a cleavage complex with DNA following treatment with VM26. The experiment is internally controlled, since

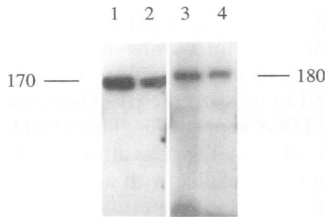

FIG. 3. Immunoblots of the 170- and 180-kDa forms of human topoisomerase II in mock-infected and infected cells. At 12 hpi, nuclei were isolated and solubilized by the direct addition of SDS. Equivalent numbers of cells from mock-infected and infected cultures were separated by SDS-PAGE as follows: lanes 1 and 3, mock-infected extracts; lanes 2 and 4, HSV-1-infected extracts. The separated proteins were then transferred to Hybond C-extra membrane and subjected to Western analysis as follows: lanes 1 and 2 were probed with p170 antibody, and lanes 3 and 4 were reacted with the p180 antibody.

formation of p170-DNA complexes is dependent upon addition of VM26 to arrest the cleavage intermediate (6). Furthermore, there was little if any difference in the amount of p170 trapped on DNA in mock-infected versus infected cells. These data argue that topoisomerase II catalytic activity (cleavage-religation on DNA) is essentially the same in mock-infected and infected cells, a result that is consistent with the mapping data in Fig. 1A. The p180 form was detectable only in those fractions containing free protein (Fig. 4B). In other words, we were unable to demonstrate in vivo trapping of endogenous p180 on DNA in either mock-infected or infected cells.

To directly demonstrate that HSV-1 DNA participated in p170-DNA complex formation, pooled DNA from the CsCl gradient fractions (containing p170-DNA complexes) was denatured and hybridized to either HSV-1 DNA or control DNA (salmon sperm). The salmon sperm DNA filters are an important control to rule out the possibility that denatured p170 protein is simply bound or trapped nonspecifically on the DNA or filter. This experiment is designed to measure (by immunoblotting) p170 that is covalently linked to HSV-1 DNA hybridizing to the DNA on filters; thus, the salmon sperm DNA control ensures that the DNA is bound by hybridization of nucleic acid. Filters were then probed with the anti-p170 antibody (Fig. 4C) to determine whether the hybrid-selected viral DNA contains p170. These data show that HSV-1 DNA was cross-linked to the p170 and that cross-linking required VM26. Parallel experiments performed with the anti-p180 antibody failed to show any cross-linking to HSV-1 or cellular DNA following treatment of mock or infected cells with VM26 (as in Fig. 4B). The absence of p180 covalently linked to DNA (Fig. 4B) would be expected if p180 was not affected by VM26 (which stabilizes the cleavage or covalent intermediate). We therefore reconstructed the analysis in vitro using partially purified p170 and p180 incubated with a plasmid (pRYG)

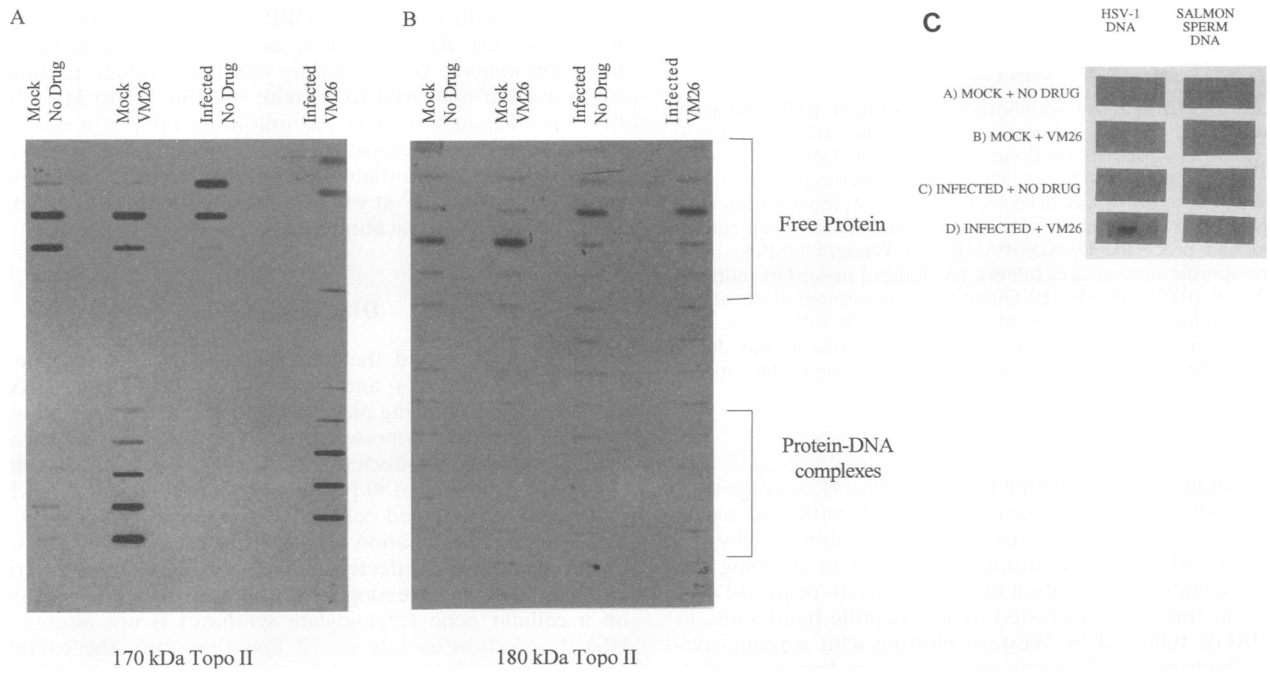

FIG. 4. Topoisomerase II binding to DNA in mock- and HSV-1-infected HeLa cells. At 12 hpi, mock- or HSV-1-infected cells were treated with 0.1 mM VM26 for 30 min and lysed with 1% Sarkosyl. Lysates were directly loaded onto CsCl step gradients as described in Materials and Methods. Gradients were fractionated, and every third fraction was applied to nitrocellulose in a slot blot manifold. Duplicate nitrocellulose filters were processed by immunoblot analysis using antibodies specific to either p170 or p180. (A) Probe with the p170 antibody; (B) probe with the p180 antibody. Column 1, mock infected, no drug; column 2, mock infected, VM26; column 3, infected, no drug; column 4, infected, VM26. The order of fractions displayed corresponds to increasing density from top to bottom; thus, free topoisomerase II (noncovalently bound to DNA) is located at the density of free protein, near the top of the gradient. Denatured topoisomerase II covalently coupled to DNA in cleavage complexes will be carried down to the CsCl step corresponding to DNA density. (C) Cross-linking of the 170-kDa topoisomerase II to HSV-1 DNA in vivo. At 12 hpi, mock- or HSV-1-infected HeLa cells were either treated for 30 min with 100 μM VM26 or left untreated, lysed with detergent, and loaded onto CsCl step gradients to prepare total DNA. The DNAs were denatured and hybridized to nitrocellulose filters containing purified HSV-1 DNA (left column) or salmon sperm DNA (right column). The filters were washed and processed as described in Materials and Methods and subjected to Western blotting analysis using the antibody specific for p170.

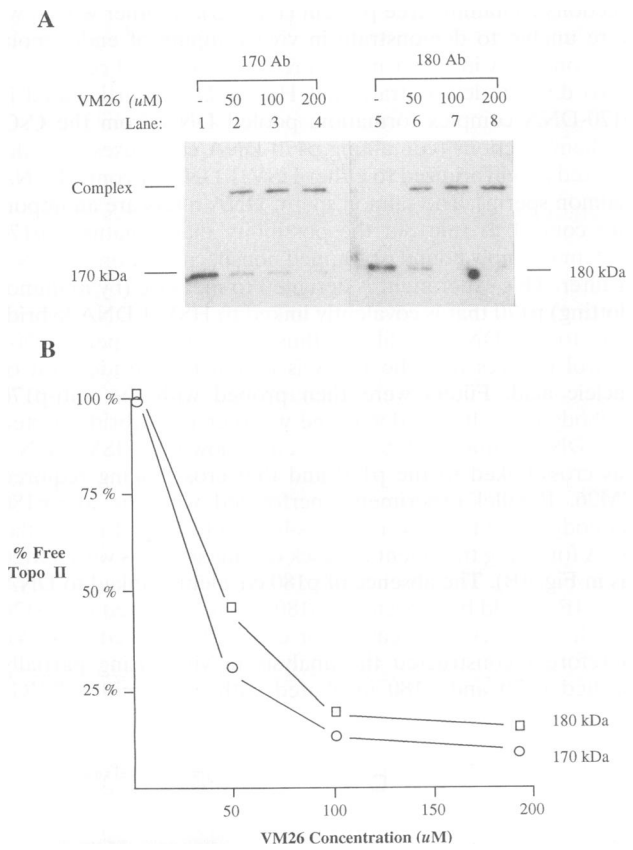

FIG. 5. VM26-induced stabilization of covalent p170 and p180 DNA complexes in vitro. A partially purified mixture of p170 and p180 HeLa isozymes was reacted with supercoiled pRYG DNA and increasing concentrations of VM26 (see Materials and Methods). To trap the enzyme in a cleavage (covalent) complex with DNA, the reactions were terminated by rapid addition of SDS. The samples were then equally divided and processed by SDS-PAGE and Western blotting using isozyme-specific antibodies as follows. (A) Lanes 1 to 4, p170 antibody; lanes 5 to 8, p180 antibody. (B) Quantitative assessment of the ability of VM26-induced stabilization of p170 and p180 with DNA. The relative amount of unbound (% Free) topoisomerase II was determined by densitometric scanning of the autoradiographic images displayed in panel A.

which contains multiple strong topoisomerase II cleavage sites (33). Reaction mixtures were incubated with increasing amounts of VM26 and the reactions were terminated by the addition of SDS (i.e., conditions that result in trapping the covalent complex). Formation of covalent p170- or p180-DNA adducts in this case was tested by polypeptide band shifts in SDS-PAGE followed by Western blotting with isozyme-specific antibody probes. The rationale is that a polypeptide-DNA adduct will display reduced electrophoretic mobility because of the covalent DNA tag. Western blots (Fig. 5A) demonstrate that both forms could be cross-linked to DNA in vitro at the lowest concentration of VM26 tested (50 μM). The drug titration profile was essentially identical for both isozymes over the range of drug tested (Fig. 5B). Therefore, the absence of p180 in protein-DNA cross-linked material from mock-infected or infected cells treated with VM26 cannot be explained by an inherent inability of VM26 to induce p180-DNA cleavage complexes.

Colocalization of the 170-kDa form of topoisomerase II and the 65-kDa DBP. The cleavage activity of p170 topoisomerase II on progeny viral genomes only suggests a role for p170 in HSV-1 DNA replication. To explore this idea, immunofluorescence was used to localize the p170 isozyme relative to sites of active HSV-1 DNA replication. The 65-kDa DBP was used as a marker for sites of DNA synthesis. This essential viral protein is known to colocalize with actively replicating HSV-1 DNA in infected cells (17). Differential detection of proteins by double immunofluorescence was accomplished by using rhodamine isothiocyanate to detect p170 and fluorescein isothiocyanate to detect the anti-65-kDa DBP. When the anti-p170 antibody was used in conjunction with both secondary antibodies, fluorescence could be detected only by using a rhodamine-isothiocyanate-specific filter (Fig. 6; compare panels C and D). Conversely, when the anti-65-kDa DBP antibody was used with both secondary antibodies, specific staining could be discriminated only by using a fluorescein isothiocyanate-specific filter (Fig. 6E and F). In the absence of either primary antibody, minimal background staining was observed (Fig. 6A and B). Immunodepletion experiments also confirm the specificity of the p170 signal, since preincubating the antibody with the peptide used as antigen completely blocked immunofluorescence (data not shown).

Figure 7 shows the simultaneous identification of subcellular positions occupied by p170 (Fig. 7A, C, E, and G) relative to those occupied by the 65-kDa DBP (Fig. 7B, D, F, and H). The p170 staining pattern at 2 hpi resembles that observed in uninfected HeLa cells (punctate and nucleoplasmic; data not shown). By 6 hpi, however, punctate p170 staining was clearly colocalized with the 65-kDa DBP (Fig. 7; compare panel C with panel D). At later times, as cytopathic effects became more prominent, colocalization was less evident (although some overlap appeared to remain; see Fig. 7E to H). From these results, we conclude the following: first, as a result of infection, p170 undergoes a clear redistribution after viral DNA replication is initiated; second, p170 clearly colocalizes with the 65-kDa DBP at what we presume to be sites of active DNA viral DNA replication.

DISCUSSION

We have examined the interaction between host type II DNA topoisomerases and viral DNA during lytic HSV-1 infection. The following pieces of evidence are consistent with the idea that host topoisomerase II levels are only slightly affected in HSV-1-infected cells. (i) Western blotting data show that p170 and p180 polypeptides are reduced somewhat (5 to 20%) in infected cells. (ii) Total enzymatic activity, as measured by decatenation activity, is nearly constant between mock-infected and infected cells (on a per-cell basis). (iii) Cleavage activity of endogenous topoisomerase II measured on a cellular gene (thymidylate synthase) is not altered by HSV-1 infection as late as 12 hpi; therefore, the catalytic activity of topoisomerase II in situ is unaltered by HSV-1. Parenthetically, host topoisomerase II (and not an HSV-1-encoded nuclease, for example) is responsible for the cleavages mapped in vivo on the basis of sequence similarity of cleavages mapped in vivo and in vitro by using purified human topoisomerase II (15). (iv) Experiments that measure formation of the covalent complex between p170 and DNA reveal similar yields of p170 in the DNA fraction from mock-infected and infected cells (Fig. 4A, lanes 2 and 4). (v) Biochemical fractionations designed to identify novel type II topoisomerase activities in HSV-1-infected cells have been uniformly negative, and although we cannot accept a negative finding as proof,

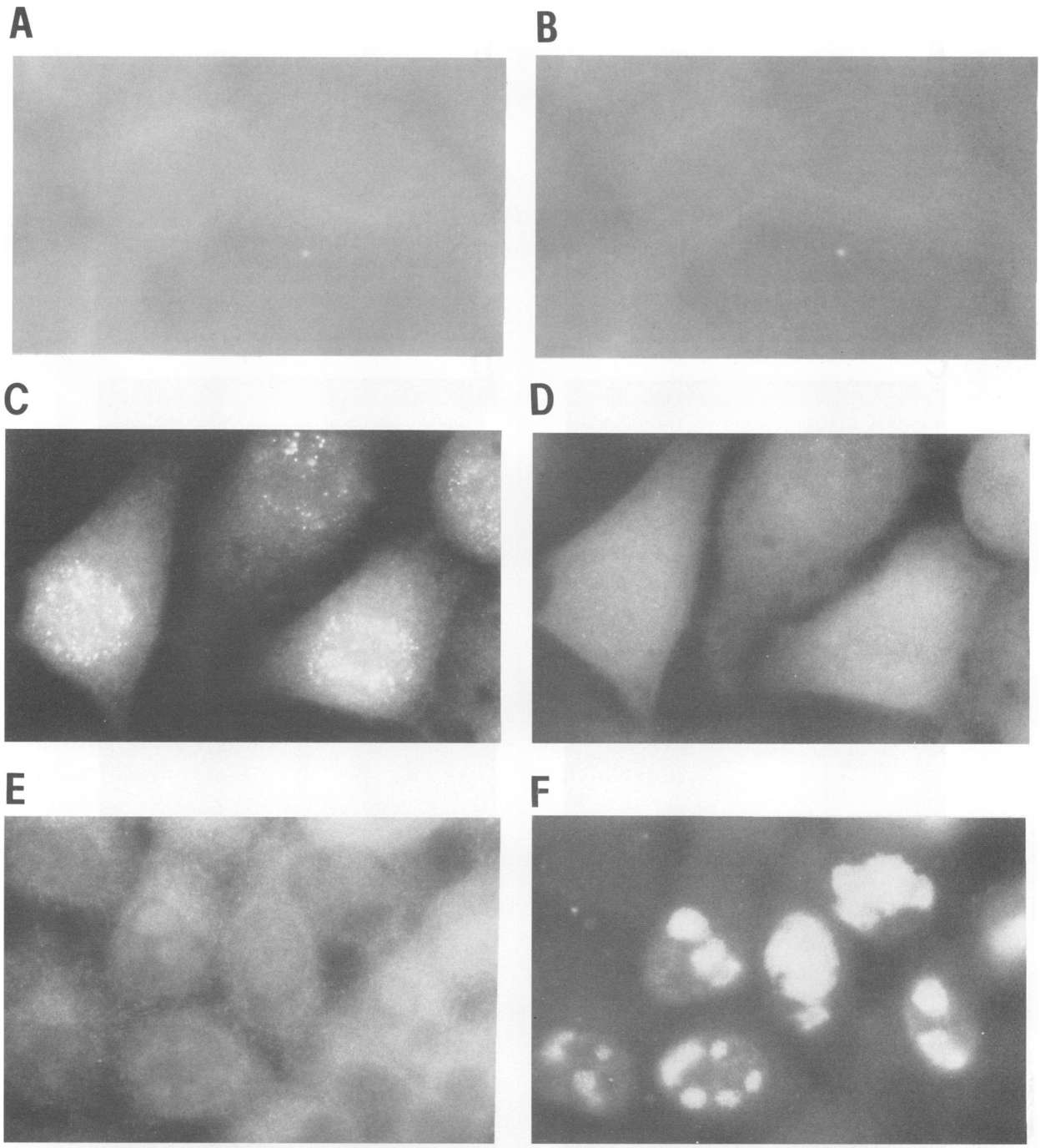

FIG. 6. Double immunofluorescent labelling of cells at 6 hpi with p170 65-kDa DBP antibodies. (A and B) No primary antibodies; (C and D) p170 antibody; (E and F) 65-kDa DBP antibody. The cells in each panel were incubated with fluorescein isothiocyanate conjugated to an anti-mouse secondary antibody and rhodamine isothiocyanate conjugated to an anti-rabbit secondary antibody. Panels A, C, and E were exposed with a rhodamine-specific filter, whereas panels B, D, and F were exposed with a fluorescein-specific filter.

at present we have no evidence that HSV-1 induces or encodes a biochemically distinct type II enzyme.

HeLa cells, the principal cell type used in this study, express both isozymes of human topoisomerase II, and both isozymes were stably present in HSV-1-infected cells. Moreover, we showed that both forms could be readily trapped in covalent complexes with DNA in vitro, while only p170 was trapped in a covalent complex with DNA in vivo. This result means that both forms are enzymatically active in vitro and engage the DNA in repeated cleavage and religation. Addition of the inhibitor VM26 impedes the religation step such that SDS denaturation traps some fraction of the enzyme in an irreversible (covalent) cleavage complex. In vivo, the same series of events causes p170 covalent cross-linking to HSV-1 and cellu-

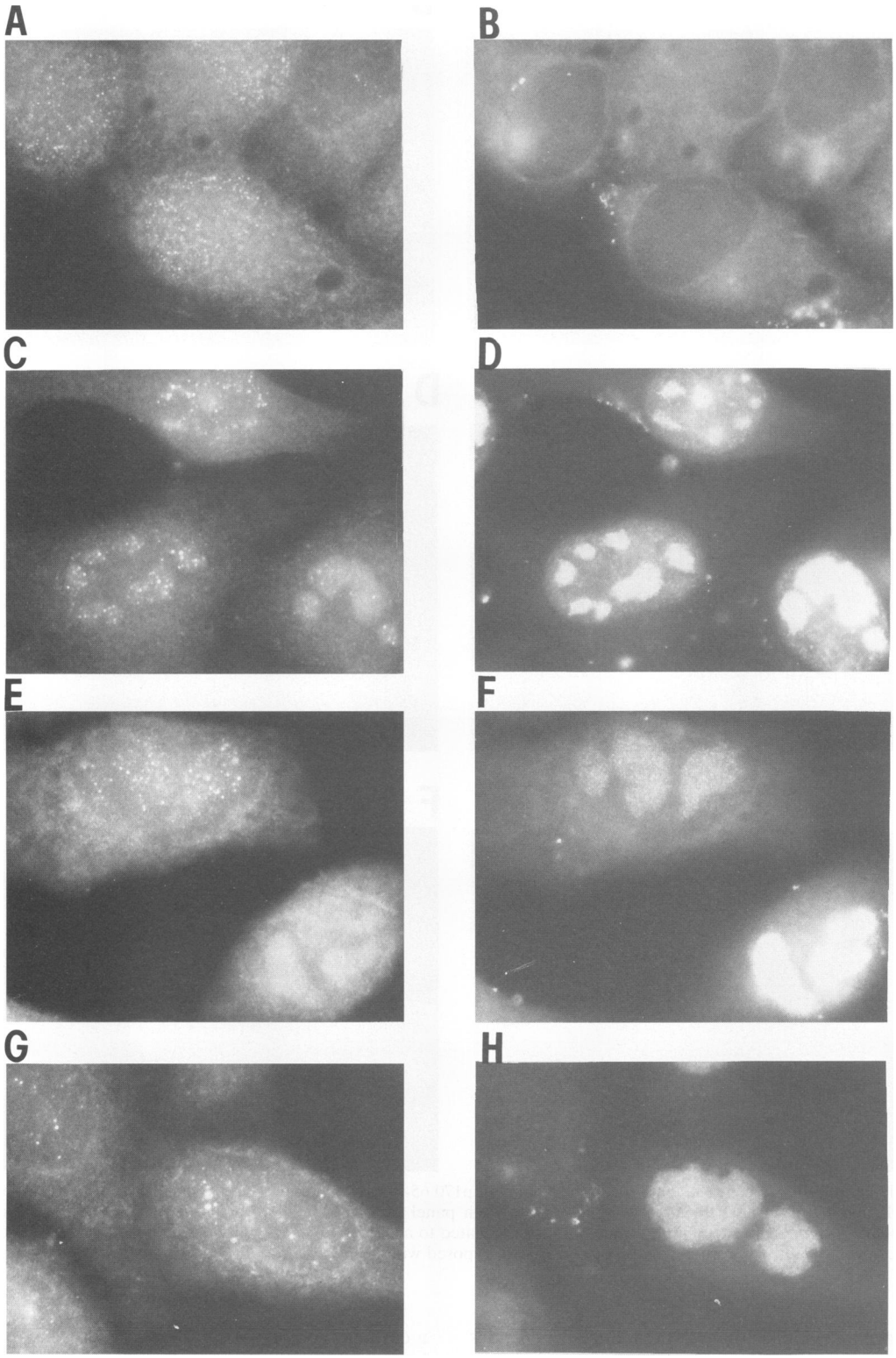

FIG. 7. Distribution of p170 in infected cells at different times postinfection. Double immunofluorescence was performed as described for Fig. 6. (A and B) 2 hpi; (C and D) 6 hpi; (E and F) 10.5 hpi; (G and H) 16 hpi. The left set of panels (A, C, E, and G) were exposed with a rhodamine-specific filter (corresponding to p170 localization); the right set of panels (B, D, F, and H) were exposed with a fluorescein-specific filter (corresponding to DBP).

lar DNA. In contrast, we were unable to demonstrate p180 covalent complexes in vivo. Nonetheless, it is possible that more sensitive techniques are required to detect interactions between p180 and HSV-1 or cellular DNA. At present, our data show that p170 interacts with HSV-1 DNA in infected HeLa cells.

Double immunofluorescence data also support the conclusion that p170 is the predominant topoisomerase II isozyme reacting with viral DNA. These experiments showed that p170 colocalized to specific subnuclear regions with the HSV-1 65-kDa DBP, a viral protein essential for DNA replication (16, 17). Colocalization of p170 and 65-kDa DBP suggests that topoisomerase II redistributes to sites of viral replication. The p170 isozyme is a component of the nuclear matrix, and there is evidence for an association between replicating viral DNA and the matrix (30). This suggests that targeting of viral DNA into the nuclear matrix would bring p170 and HSV-1 DNA into close proximity; however, our immunolocalization data additionally suggest an active redistribution of preexisting topoisomerase II into these sites. Targeting of p170 into viral replication compartments may in fact explain why there is preferential cleavage of progeny or replicated viral DNA molecules (i.e., after initiation of HSV-1 DNA synthesis) (15). Given that the total amount of p170 is unchanged between 2 and 12 hpi, enhanced cleavage of progeny genomes may be due to clustering of p170 at replicative sites of HSV-1 DNA.

The underlying mechanism that drives redistribution of p170 from prereplicative to replicative viral compartments is unknown but may be related to findings in recent studies suggesting that topoisomerase II has a high affinity for nodes of DNA crossovers (where one duplex segment contacts another) (20, 47). Previously, we demonstrated that p170 forms complex aggregates with G-rich DNA sequences (like HSV-1) (7). If such DNA structural features arise in replicating HSV-1 DNA, perhaps p170 recognizes these and becomes firmly bound. This association could be functional as well, since topoisomerase II may provide a mechanism to aid in resolving large interlocked complexes of progeny viral DNA molecules, antecedent to maturation and assembly. Genetic and biochemical studies reveal that topoisomerase II performs an essential step in decatenating and resolving daughter chromatids in the host cell (19, 39).

Clearly, more studies will be necessary to determine the precise function of the p170 in HSV-1 replication. Additional possibilities include relief of torsional stress produced during viral DNA replication (5, 11) or participation in recombination (2, 9), a process which is very active in infected cells following the initiation of HSV-1 DNA replication. Alternatively, host type II topoisomerases may be inconsequential for HSV-1 replication, given that the virus can replicate (although with lower yields) in G_1-arrested cells (28a) that possess significantly lower levels of p170 (18). Colocalization and cleavage of viral DNA may be coincidental because topoisomerase II has an affinity for DNA crossover regions (7, 20, 47), which are likely to be abundant at late times postinfection. It is also possible that the association is mediated by protein-protein interactions between p170 and some other viral or cellular protein which actively enters viral replication compartments. These possibilities are currently under investigation.

Additional work will be required to sort out the functional basis of these findings; however, it is significant that a host protein (p170) can be subverted into a viral DNA damaging agent with antitumor drugs like VM26. These findings suggest new avenues of antiviral drug development. Although p170 is present in uninfected cells, the fact that it redistributes and seeks out viral replication complexes suggests that at the subcellular level, DNA damage could be selectively targeted to viral DNA. Since we have shown that the p170 isozyme is responsible for the HSV-1 DNA damage, future antiviral strategies should focus on drugs which are more selective for p170 rather than p180.

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