Human Cytomegalovirus Induces Expression of Cellular Topoisomerase II

JOHN D. BENSON^{1†*} AND E.-S. HUANG^{1,2}

Curriculum in Genetics¹ and Departments of Medicine and Microbiology/Immunology,² University of North Carolina, Chapel Hill, North Carolina 27599

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Previous work from our laboratory has suggested that topoisomerase II is required for replication of human cytomegalovirus (HCMV). In assays of confluent human embryonic lung cells infected with HCMV, topoisomerase II inhibitors exhibited an irreversible inhibition of viral DNA replication. However, Northern (RNA blot) and Western (immunoblot) analyses of confluent uninfected human embryonic lung cells detected very low levels of cellular topoisomerase II RNA and protein. Quantitation of human topoisomerase II RNA and protein levels at various times after HCMV infection revealed that HCMV induces increased intracellular levels of both topoisomerase II RNA and protein. Such accumulation began at early times of infection, continued through late in infection, and was not reduced by inhibition of viral DNA synthesis. This is the first report of such induction by a viral infection. Topoisomerase II was also detected in isolated HCMV virions.

DNA topoisomerases are enzymes that modulate the topological state of DNA. Type II topoisomerases do this by a strand-crossing reaction involving a double-strand break in the DNA helix. This gives topoisomerase II the unique ability to catalytically relax, unknot, and decatenate covalently closed DNA molecules (45). Topoisomerase II has now been shown to be essential for replication of cellular (9, 21, 22, 29, 44) as well as viral DNA (11, 32, 35-37, 47).

We have recently demonstrated that topoisomerase II inhibitors prevent replication of human cytomegalovirus (HCMV) DNA in tissue culture (2). In these experiments, confluent human embryonic lung (HEL) primary fibroblasts were infected with HCMV and treated with two classes of topoisomerase II inhibitors. Both classes, intercalative (m-AMSA) and non-intercalative (VM-26), were effective in eliminating detectable replication of viral DNA.

Many groups have reported an association between intracellular levels of topoisomerase II and the proliferative state of the cells (10, 19, 27, 28, 40). In general, topoisomerase II is in greater abundance in proliferating cells than in quiescent cells. More recently, this phenomenon has been documented in human skin fibroblasts, in which the topoisomerase II level was shown to be sensitive to serum deprivation and cell density (24). Indeed, by immunofluorescence assay, positive staining for topoisomerase II was only observed in fibroblasts grown at low densities and in high serum concentrations.

Although topoisomerase II inhibitors exhibit an unmistakable inhibitory effect on HCMV DNA synthesis in confluent primary HEL fibroblasts, this effect could not be readily accounted for when considered in the context of the results described above. Namely, the cells in which we observed an antiviral effect by topoisomerase II inhibitors, according to the results described above, would normally not contain topoisomerase II. There is a strong correlation between the amount of topoisomerase II activity and the effects of topoisomerase II inhibitors (7, 8, 25, 31). Therefore, in order to more fully explain the observed antiviral effects of topo-

isomerase II inhibitors, we reasoned that topoisomerase II levels in HCMV-infected cells must increase in response to infection. Such an increase could be via expression of a virus-encoded topoisomerase II or by induction of host cell topoisomerase II expression.

In order to address this issue, we undertook hybridization studies using a universal topoisomerase II probe, which is a mixed oligonucleotide that includes each possible codon for the conserved amino acid sequence MIMTDQD. This probe has been used successfully to clone a number of other topoisomerase II genes (43; J. C. Wang, personal communication). However, it failed to detect cross-hybridization to sequences within the HCMV genome. Within the limits of this assay, we therefore concluded that HCMV probably does not encode a topoisomerase II of its own (J. D. Benson, Ph.D. dissertation, University of North Carolina, Chapel Hill, 1989).

Instead, HCMV infection leads to a dramatic increase in the steady-state levels of cellular topoisomerase II RNA and protein. Topoisomerase II is also detectable in isolated HCMV virions. In concurrence with previously published work (24), topoisomerase II RNA and protein were present at very low levels in confluent HEL cells.

MATERIALS AND METHODS

Cells and virus. HEL cells were maintained and infected as previously described (26). Confluent cells were infected with HCMV (Towne strain, passage 40) at a multiplicity of infection of 2 to 3 infectious units per cell.

Northern (RNA blot) analysis. Total RNA (20 µg) was isolated by guanidine isothiocyanate-CsCl extraction (4), suspended in TE (10 mM Tris [pH 7.8], 1 mM EDTA), ethanol precipitated, and stored at -20°C in TE until use. For formaldehyde-1.2% agarose gel electrophoresis and Northern analysis, 20 µg of total RNA was loaded per lane, with duplicate lanes for ethidium bromide (EtBr) staining of rRNA. After transfer overnight, blots were baked and pre-hybridized in 25 mM KPO₄-5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-5× Denhardt solution-50 µg of sonicated salmon sperm DNA per ml-50% formamide overnight at 42°C. Overnight hybridization was at the same temperature in the same solution, except for the addition of

* Corresponding author.

† Present address: Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, MD 20892.

FIG. 1. The previously described 2.4-kilobase partial cDNA probe hTOP2-1 was used as a probe for Northern blots (43). Below this is shown a schematic representation of the 6.2-kilobase cDNA encoding human topoisomerase II. The length of the 5'-untranslated leader sequence has not been previously determined. The oligonucleotide probe used for topoisomerase II primer extension is shown below the 6.2-kb cDNA. This 27-mer corresponds to the noncoding cDNA strand from +10 to +37 of the open reading frame encoding human topoisomerase II.

10% dextran sulfate. A topoisomerase II cDNA probe (Fig. 1) was labeled by nick translation, and 5×10^5 cpm of probe per ml was added directly to the prehybridization solution.

Primer extension. Primer extension was carried out by using the 27-base oligonucleotide 5'-CATATTTTCATTTCAGGCTGCAATGG-3'. This sequence corresponds to the noncoding strand of the previously described human topoisomerase II open reading frame (43), originating from +13 to +40 relative to the ATG initiation codon (Fig. 1). The 30-base oligonucleotide 5'-GAGCGCGCGATATCATCATCCATGGTGAG-3' derived from cDNA sequences described by Gunning et al. (17) was used for β -actin primer extension. Both primers were synthesized on an Applied Biosystems synthesizer, purified by polyacrylamide denaturing gel electrophoresis, and end labeled by using 10 U of polynucleotide kinase (Promega Biotec) and [γ - 32 P]ATP (Dupont, NEN Research Products). End-labeled primer (5×10^5 cpm) was annealed to 30 μ g of total RNA overnight at 30°C in 1 $\times$ hybridization solution (80% formamide, 40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 6.4], 400 mM NaCl, 1 mM EDTA). After ethanol precipitation, reverse transcriptase reactions were carried out in a final volume of 25 μ l by using 30 U of avian myeloblastosis virus reverse transcriptase at 42°C for 90 min in 50 mM Tris chloride (pH 8.0)–5 mM MgCl₂–5 mM dithiothreitol–50 mM KCl–50 U RNasin–50 μ M deoxynucleoside triphosphates. Reactions were stopped by the addition of 1 μ l of 0.5 M EDTA, phenol–chloroform extracted, ethanol precipitated, washed in 70% ethanol, and run on a 6% polyacrylamide sequencing gel.

Western immunoblot analysis. Proteins from 2×10^6 cells were harvested by washing cells twice in 10 ml of phosphate-buffered saline (PBS), scraping them into 40 mM Tris chloride (pH 7.4)–1 mM EDTA–150 mM NaCl, pelleting the cells by centrifugation at 4°C for 5 min, suspending the cell pellet in 50 μ l of PBS, and then adding 50 μ l (2 $\times$) of sodium dodecyl sulfate–polyacrylamide gel electrophoresis loading dye. A small sample was saved for protein concentration determination with a Bradford assay kit (Bio-Rad Laboratories). Protein (100 μ g) was loaded on a 4% stacking, 7% running sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. Gels were electroblotted overnight onto nitrocellulose in 1 $\times$ transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol, pH 8.0) at 0.2 A. After transfer, the membrane was blocked by using 3% Blotto (3% [wt/vol] Carna-

tion evaporated milk in 1 $\times$ PBS). Antibody reactions were with a 1:100 dilution of rabbit polyclonal antiserum to the carboxyl-terminal one-third of human topoisomerase II in 10 ml of 3% Blotto with shaking at room temperature for 2 h. After being washed three times for 10 min each with PBS, anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Promega Biotec) was added (1:7,500 working dilution) in 3% Blotto. After 2 h of shaking at room temperature, the filter was again washed three times with 10 ml of PBS, and the alkaline phosphatase reaction was carried out as recommended by the manufacturer.

Isolation of HCMV virions. HCMV virions were purified from extracellular culture fluid from HEL cultures infected with HCMV. Medium was cleared of cells by centrifugation at $6,000 \times g$ for 20 min at 4°C. Polyethylene glycol (PEG 6000) was then added to a final concentration of 10% (wt/vol), and the solution was chilled on ice overnight. Virus was then pelleted by centrifugation at $6,000 \times g$ for 60 min and suspended in ice-cold Tris-buffered saline. The suspension was dispersed by vortexing and layered onto a gradient of 10 to 50% sucrose. After centrifugation at $150,000 \times g$ in a Beckman SW27 rotor for 1 h at 4°C, the opaque virus band was collected and dialyzed against Tris-buffered saline.

RESULTS

Accumulation of cellular topoisomerase II transcripts in HCMV-infected cells. Previous work from our laboratory has demonstrated that HCMV replication requires topoisomerase II, even in confluent stationary primary fibroblasts (2). Others have demonstrated that intracellular levels of topoisomerase II protein and enzymatic activity correlate tightly with cell division; dividing primary cells contain high levels of topoisomerase II, whereas stationary and differentiated cells do not (3, 18, 19, 24, 40). By measuring the abundance of topoisomerase II RNA and protein in infected cells at various times after infection, we hoped to determine whether HCMV might fulfill its need for topoisomerase II by inducing expression of the cellular gene.

The result of a Northern blot of total RNA from HCMV-infected HEL cells at various times after infection is shown in Fig. 2. Although low steady-state levels of topoisomerase II RNA were detectable in mock-infected control cells, a significant increase had occurred by 48 h after infection. Accumulation of rRNA, which was detected by the cross-

FIG. 2. Upper panel: total RNA was harvested from HCMV-infected HEL cells at different times after infection (HPI, hours postinfection). RNA (20 μ g per lane) was loaded in a 1.2% agarose-formaldehyde gel. The Northern blot was probed with a nick-translated topoisomerase II cDNA probe (Fig. 1a). Lower panel: the same blot after stripping and reprobing for human β -actin transcripts. Laser densitometry was used to quantitate the topoisomerase II and β -actin bands, the latter of which was used as a normalization value when determining the fold topoisomerase II induction described in the text. kb, Kilobase.

reaction of vector sequences (data not shown), is a previously documented response to HCMV infection of fibroblasts (39). This disallowed its use as a quantitation reference. According to laser densitometry quantitation and signal normalization to blots reprobed with β -actin, a transcript not induced by HCMV infection (5), we estimate this increase to be approximately sevenfold over mock-infected cells. The timing of maximal topoisomerase II RNA accumulation is in agreement with previous data indicating that the primary requirement for topoisomerase II in the HCMV infectious cycle occurs at early and late times of infection, the times of maximal DNA replication (2). The steady-state levels of topoisomerase II RNA in unsynchronized HeLa cells is quite high (Fig. 2), but transcripts from both these and infected HEL cells were roughly the same size (6.2 kilobases). This agrees with the previously reported size of the human topoisomerase II transcript (43).

The results of a quantitative primer extension experiment which also indicates the temporal accumulation of topoisomerase II transcripts in HCMV-infected cells are shown in Fig. 3, upper panel. (Lane 1 was overloaded in order to visualize the low level of topoisomerase II primer extension products in mock-infected cells.) Greater amounts of end-labeled oligonucleotide primer did not increase the intensity of the primer extension product band, indicating that primer was not limiting in the assay. Quantitation of such primer extension was done by normalizing to bands produced by primer extension of β -actin transcripts (Fig. 3A, lower panel). These results (Fig. 3B) indicated a greater than 10-fold increase of topoisomerase II RNA levels in infected cells 72 h postinfection compared with mock-infected con-

FIG. 3. (A) Upper panel: primer extension of total RNA from infected HEL cells at various times after infection (HPI, hours postinfection). A topoisomerase II-specific end-labeled oligonucleotide primer plus 30 μ g of RNA was used per primer extension reaction. Primer extension products were analyzed on a 6% polyacrylamide sequencing gel. Lane 1 was overloaded (60 μ g RNA) in order to visualize the 418-nucleotide topoisomerase II primer extension product. Lower panel: parallel primer extensions obtained by using a β -actin-specific primer for normalization. (B) Primer extension products were quantitated by laser densitometry, using β -actin signal intensity for normalization, and graphed as shown.

trols. This value was reproducible and was in close agreement with that observed by quantitative Northern blot analysis.

The 418-nucleotide primer extension product predicts a 5' untranslated topoisomerase II leader sequence of 343 nucleotides. This is in general agreement with the predicted leader sequence size of the human topoisomerase II mRNA, which by Northern analysis of transcripts from HeLa cells has a predicted 5' untranslated leader sequence several hundred nucleotides in length (43).

Temporal regulation of HCMV gene transcription falls into three major categories. Immediate-early is the first burst of viral transcription in the infected cell. This relies upon transcription factors provided by the host cell and, possibly, by the virion itself. Many of the gene products from this temporal class are transcription factors, which are thought to be capable of activating transcription of both cellular genes and viral genes of later temporal classes. Early genes are characterized by their requirement for de novo protein synthesis before their transcriptional activation, but they do not depend upon viral DNA synthesis for their transcription or translation. Genes in this class include the majority of viral and cellular gene products involved in viral DNA replication. Viral late genes are distinguished from early genes by the fact that they require replication of the viral genome before their appearance in the infected cell. DHPG [9(1,3-dihydroxy-2-propoxymethyl)-guanidine], an inhibitor of HCMV replication which targets the virus-encoded DNA polymerase (26), did not affect accumulation of the topoisomerase II transcript (Fig. 4). This result, as well as the temporal appearance of topoisomerase II RNA, would suggest that its regulation resembles that of early viral gene products. Indeed, this result prompts speculation that the HCMV immediate early protein products, which are documented promiscuous transcriptional activators of both viral and cellular genes (30), may be responsible for the observed activation effects.

Accumulation of topoisomerase II protein during HCMV infection. Although other workers have demonstrated differential intracellular levels of topoisomerase II in dividing versus stationary cells, as well as constitutively high levels of topoisomerase II in transformed cells, the true nature of this regulation remains unclear. For this reason, it was possible that the observed accumulation of topoisomerase II transcripts in HCMV-infected cells would not be reflected in protein accumulation. Many HCMV early and late genes are posttranscriptionally controlled (13–15).

Using polyclonal rabbit antiserum to the carboxyl-terminal one-third of topoisomerase II, we measured the intracellular levels of topoisomerase II at various times after infection with HCMV. Topoisomerase II was virtually undetectable in uninfected confluent HEL cells (Fig. 5). However, topoisomerase II accumulated in infected cells with kinetics similar to that of the RNA levels. Indeed, by late times of infection, topoisomerase II constituted a higher percentage of total intracellular protein than was present in unsynchronized HeLa cells. This demonstrates a remarkable induction of intracellular enzyme levels, which is reflected in RNA abundance as well. Quantitation of the relative level of topoisomerase II protein induction was difficult because of the virtually undetectable levels in mock-infected cells. However, the magnitude of protein induction by HCMV infection appears to be at least as great as the increase in topoisomerase II RNA levels in infected cells. Although we do not attempt to rule out the possibility of posttranscriptional control, we feel that these data strongly suggest a role for transcriptional induction.

It is not clear why two closely migrating topoisomerase II protein bands are clearly visible in the infected cells, whereas only the upper band was observed in HeLa cells. Hseih observed a similar set of bands in isolation of topoisomerase II from *Drosophila* embryos (23). In that work, protein from both bands showed identical protease digestion patterns and bound to DNA. Similar topoisomerase II protein patterns have been observed in chicken fibroblasts as well (19). It is interesting that in our experiments, Western

FIG. 4. Total RNA was harvested from mock-infected cells and from infected cells 72 h postinfection (HPI). Quantitative primer extension was carried out as described for Fig. 3, using 30 μ g of RNA per reaction. One infected-cell flask was treated with DHPG, an inhibitor of the HCMV DNA synthesis, for the entire period of infection (right lane). nt, Nucleotide.

analysis of protein extracts from HeLa cells (Fig. 5) and from HCMV-transformed fibroblasts (data not shown) revealed only the upper topoisomerase II band. We are currently investigating the significance of these observations.

Topoisomerase II was also detected in isolated HCMV virions (Fig. 5). Cellular topoisomerase II has previously been found in isolated simian virus 40 virions (L. F. Liu, personal communication). This may reflect the role of topoisomerase II in the terminal replicative phases of these respective genomes immediately before packaging. Alternatively, topoisomerase II may be directly involved in modulating the torsional status of viral DNA during the packaging process itself.

DISCUSSION

The importance of topoisomerase II in DNA replication has been documented in a number of cellular and viral systems (9, 11, 21, 22, 32, 35–37, 44, 47). Whereas either topoisomerase I or II may serve as the swivel to alleviate torsional strain produced by the movement of replication forks, the type II enzyme is required for the separation of newly replicated chromosomes as well as for decatenation of intertwined daughter DNA strands. The abundance of topoisomerase II in a variety of transformed cells has made this enzyme an attractive target for cancer chemotherapy (25).

Little is known about the control of topoisomerase II gene expression. Several groups have demonstrated that intracellular levels of topoisomerase II are tightly linked to the proliferative or differentiation status of the cell. Duguet et al. demonstrated that after partial hepatectomy of rats, there was an increase in topoisomerase II activity during the subsequent regenerative process (10). Using guinea pig lymphocyte stimulation with the mitotic agent concanavalin A, Tandau et al. later demonstrated that topoisomerase II was stimulated in parallel with DNA synthesis (40). Intracellular topoisomerase II levels have also been shown to be

FIG. 5. (A) Total proteins were harvested from HCMV-infected HEL cells at various times after infection. Protein (100 μ g per lane) was loaded and run on a 4% stacking–7% separating sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After electroblotting overnight, blots were reacted with anti-topoisomerase II rabbit antiserum and then with anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. Topoisomerase II appears as a doublet at 170 kilodaltons (kDa), its predicted size. Total protein (100 μ g) from HeLa cells is also shown. (B) SDS-PAGE-Western blot analysis of topoisomerase II from HCMV-infected cells as well as from isolated HCMV virions.

tightly linked to the mitotic state of cells. Hsiang et al. (24) have shown that the levels of topoisomerase II in primary human fibroblasts correlate well with cell proliferation, with high intracellular levels during the late G1, S, G2, and M phases of the cell cycle. However, upon progression from mitosis into G1, a drastic decrease in intracellular topoisomerase II levels occurred. Similar results were obtained with chicken embryo fibroblasts by Heck et al., who observed that topoisomerase II accumulation began with the start of DNA replication and that accumulation continued through the S and G2 phases of the cell cycle (19).

Our study confirms the finding that growth-arrested fibroblasts have low intracellular levels of topoisomerase II. This paucity is reflected at both the RNA and protein levels. In addition, topoisomerase II RNA and protein levels increase in response to infection with HCMV. Although others have reported stimulation of topoisomerase II activity in fibroblasts stimulated by epidermal growth factor (27) and serum (24), this is the first report of induction by a viral infection. In addition, our data suggest that this induction is at least in part due to an increase in the intracellular levels of topoisomerase II RNA. This effect is not hindered by DHPG, a specific inhibitor of the HCMV-encoded DNA polymerase. Thus, it appears that topoisomerase II induction by HCMV can occur independently of viral DNA replication.

HCMV induces a variety of cellular genes that may be required for viral DNA replication (5, 12, 20). The immediate-early genes of HCMV are strong promiscuous transcrip-

tional activators of a variety of cellular and viral promoters (6, 30). The kinetics of topoisomerase II induction by HCMV infection, as well as the fact that inhibition of viral DNA replication has no effect upon topoisomerase II RNA or protein accumulation, would be consistent with a role for immediate-early transcriptional activation. Experiments are now underway to clone and characterize the topoisomerase II promoter and to test its responsiveness to these gene products.

Alternatively, HCMV infection may act in an indirect manner to activate topoisomerase II expression. It has been suggested that HCMV infection metabolically stimulates cells in a manner similar to that of progression into the mitotic phase of the cell cycle (16, 38). This is certainly borne out here, since HCMV infection causes accumulation of topoisomerase II, a very specific marker for cell division. Schickedanz et al. have described *c-fos* induction and cellular DNA synthesis in response to microinjection of pp89, the major immediate-early gene product of murine cytomegalovirus (34). *c-fos* expression appears to be a prerequisite for entry into the cell cycle. Although the effects of the HCMV immediate-early gene products on *c-fos* and other cellular proliferation-linked genes are not known, they may act in a similar manner. Thus, topoisomerase II induction could be a direct effect of stimulation by HCMV immediate-early genes or an indirect effect of HCMV immediate-early genes on cellular genes like *c-fos*.

It is interesting that we detected a 170-kilodalton topoisomerase II doublet in HCMV-infected cells but not in HeLa cells (Fig. 5), in which only the upper band of the doublet was observed. HCMV-transformed fibroblasts also displayed only the upper topoisomerase II band (unpublished results). This exactly reflects results by Heck et al., who saw an identical doublet in chicken embryo fibroblasts but only one reactive band in transformed 293 cells (19). Results like these may reflect altered modification or proteolysis of topoisomerase II in primary versus transformed cells. Such alterations in its processing have been previously proposed (1, 19, 33).

Very little is known about the regulation of topoisomerase II expression. However, aberrant expression of topoisomerase II is a common phenotype of transformed cells. This work represents the first report of stimulated cellular topoisomerase II expression in response to viral infection. Furthermore, this induction appears to be inappropriate, in that the confluent primary cells in which this effect was observed normally express very low levels of topoisomerase II. It will be interesting to see whether other DNA viruses, including those associated with transformation, have a similar effect upon the regulation of topoisomerase II.

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