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# DNA Synthesis in Polyoma Virus Infection

## V. Kinetic Evidence for Two Requirements for Protein Synthesis During Viral DNA Replication

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Protein synthesis in polyoma virus-infected cells was inhibited by 99% within 4 min after exposure to 10 $\mu$g of cycloheximide per ml. Subsequent to the block in protein synthesis, the rate of viral DNA synthesis declined via inhibition of the rate of initiation of new rounds of genome replication (Yu and Cheevers, 1976). This process was inhibited with complex kinetics: within 15 min after the addition of cycloheximide, the rate of formation of closed-circular viral DNA was reduced by about one-half. Thereafter, DNA synthesis in cycloheximide-treated cells declined more slowly, reaching a level of 10% of untreated cells only after approximately 2 h. Protein synthesis was also required for normal closure of progeny form I DNA: in the presence of cycloheximide, DNA synthesis was diverted from the production of form I to form Ic, a monomeric closed-circular DNA component deficient in superhelical turns (Yu and Cheevers, 1976). Form I is replaced by Ic with first-order exponential kinetics. It is concluded that at least two proteins are involved in the control of polyoma DNA replication. One is apparently a stoichiometric requirement involved in the initiation step of viral DNA synthesis, since this process cannot be maintained at a normal rate for more than a few minutes in the absence of protein synthesis. The second protein requirement, governing the closure of newly synthesized progeny DNA, is considered distinct from the "initiation" protein on the basis of the kinetic data.

Previous work has examined in detail the relationship between protein synthesis and the replication of papovavirus DNA. Polyoma and simian virus 40 DNA synthesis are inhibited by treatment of infected cells with either puromycin or cycloheximide (1, 2, 13). The amount of polyoma DNA made subsequent to inhibition of protein synthesis is limited solely by the rate of initiation of new rounds of genome replication (18). Viral DNA molecules already initiated at the time of addition of cycloheximide, as well as the reduced number of molecules initiated after protein synthesis has been blocked, finish replication normally to yield closed-circular progeny DNA. Thus, the elongation of nascent viral DNA fragments into strands of unit genome length and the conversion of these replicating molecules into closed-circular DNA do not require concurrent protein synthesis. However, the progeny DNA formed in the absence of protein synthesis has an altered tertiary structure, characterized by a two-thirds reduction in superhelicity as compared to form I DNA (1, 4, 17). This DNA, which we have termed form Ic (17), is a monomeric closed-circular DNA species that exhibits a lower superhelix density than form I, corresponding to $\Delta\sigma_0 = 0.0195$ by isopycnic analysis in cesium chloride-propidium diiodide. Form Ic is synthesized on pre-existing form I templates without the intervention of progeny form I as an intermediate, resulting therefore from an alteration of the closure of newly replicated DNA.

The purpose of the present study was to examine the kinetics of inhibition of viral DNA synthesis and the rate at which form I DNA is replaced by form Ic in cycloheximide-treated cells. The results indicate that the rate of formation of closed-circular DNA (forms I and Ic) is reduced by one-half within only 10 to 15 min after the inhibition of protein synthesis. In contrast, protein synthesis must be arrested for 90 min to allow replacement of the new synthesized form I molecules by Ic. These findings are interpreted to mean that the initiation step in viral DNA synthesis and the normal closure of newly replicated molecules require the synthesis of two different proteins.

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Bacteriology and Immunology, University of Western Ontario, in partial fulfillment of requirements for the Ph.D. degree.)

## MATERIALS AND METHODS

The methods used for this work are described in the accompanying paper (17) with the following additions.

Total closed-circular polyoma DNA was analyzed by velocity sedimentation in alkaline sucrose gradients. For this, appropriate neutral sucrose gradient fractions containing viral DNA were combined, and the DNA was precipitated in 2.5 volumes of cold ethanol. The DNA was dissolved in 0.3 M NaCl-0.001 M EDTA-0.01 M Tris, pH 8.1, denatured by the addition of 0.1 volume of 1 N NaOH-0.01 M EDTA, and sedimented in 15 to 30% (wt/wt) sucrose gradients in 0.5 M NaCl-0.25 N NaOH-0.001 M EDTA-0.1% (wt/vol) *N*-lauroyl sarcosine (Spinco SW27.1 rotor, 26,500 rpm, 8 h, 32 C). Under these conditions, both form I and Ic polyoma DNA sediment at 53*S* (1, 4).

Protein synthesis was measured by incorporation of L-[$^3$H]amino acids (New England Nuclear) into material insoluble in hot 5% trichloroacetic acid. Sodium dodecyl sulfate lysates of cells were mixed with an equal volume of 10% trichloroacetic acid, and the mixture was heated for 20 min at 90 C. The acid-insoluble fraction was then collected by filtration onto membranes (Millipore) (0.45 $\mu$m) and assayed for radioactivity as previously described (6).

## RESULTS

**Kinetics of inhibition of protein and viral DNA synthesis by cycloheximide.** The experiment described in Fig. 1a shows that cycloheximide, at a concentration of 10 $\mu$g/ml, rapidly blocks the cumulative synthesis of protein in polyoma-infected cells. The rate of incorporation of [$^3$H]amino acids was reduced by 94% within 1 min after the addition of cycloheximide and by 99% within 4 min (Fig. 2).

To measure the inhibition of viral DNA synthesis, medium containing [$^3$H]thymidine (TdR) with or without cycloheximide was added to infected cultures at 24 h postinfection. At various times thereafter, treated and untreated cells were harvested, and labeled viral DNA was separated from high-molecular-weight cellular DNA by sedimentation in neutral sucrose gradients as previously described (6, 17). The viral DNA was then isolated from appropriate neutral gradient fractions, and closed-circular components were separated by alkaline velocity sedimentation analysis. Under these conditions, both form I and form Ic (synthesized in cycloheximide-treated cells) are resistant to alkali-induced strand separation and sediment at 53*S* (1, 4).

Figure 3 shows representative sedimentation profiles. The main point to be made is that, at

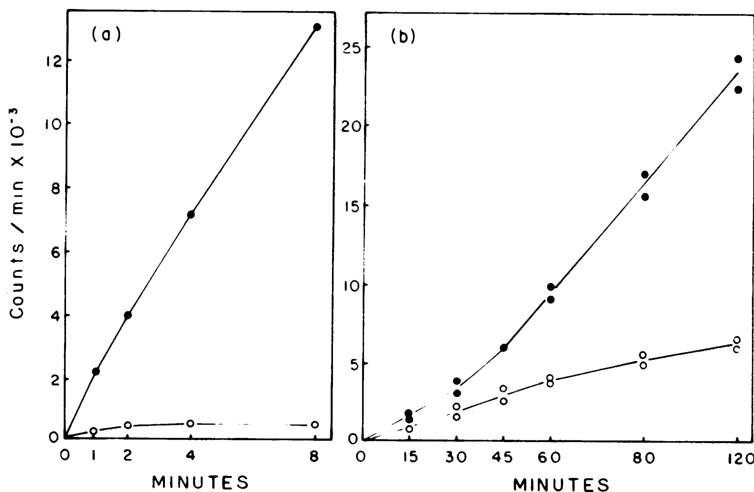


FIG. 1. *Effect of cycloheximide on the cumulative synthesis of protein and closed-circular viral DNA in polyoma-infected cells. (a) Protein synthesis: Infected cultures were labeled at 24 h postinfection with [$^3$H]amino acids (4 $\mu$Ci/ml) with or without cycloheximide. Incorporation into the hot trichloroacetic acid-insoluble fraction was followed for 8 min. (●) Untreated cultures; (O) cycloheximide-treated cultures. (b) DNA synthesis: Infected cultures were labeled at 24 h postinfection with medium containing [$^3$H]TdR (25 $\mu$Ci/ml) and 0.1 $\mu$g of unlabeled TdR with or without cycloheximide per ml. Incorporation into closed-circular viral DNA was measured after the indicated times by the procedures described in Fig. 1. (●) Untreated cultures; (O) cycloheximide-treated cultures.*

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all three time intervals (15 min, Fig. 1a; 30 min, Fig. 1b; 60 min, Fig. 1c), less radioactively labeled 53*S* DNA was made in cycloheximide-treated cells than in untreated cells. Figure 1b shows the cumulative synthesis of closed-circular viral DNA as determined by the methods described in Fig. 3. As expected, the incorporation of [$^3$H]TdR into viral DNA between 24 and 26 h postinfection increased cumulatively at a progressively more rapid rate in untreated cells. Labeled DNA accumulated much more slowly in cycloheximide-treated cells.

In comparison to untreated controls, the rate of accumulation of viral DNA in the presence of cycloheximide was progressively reduced with time. This is more evident by the data of Fig. 2, which illustrates the slope of cumulative incorporation curves in treated cells as a function of that in untreated cells. From this calculation, it is obvious that viral DNA synthesis was inhibited by cycloheximide with complex kinetics. The rate of formation of 53*S* DNA was reduced by 40 to 50% within 15 min after the addition of cycloheximide. Thereafter, the rate of DNA synthesis declined more slowly, reaching a level of 10% of that in untreated cells only after about 2 h.

One explanation for suppression of DNA synthesis by cycloheximide is that this drug causes a reduction of deoxyribonucleotide pools, so that the concentration of TdR nucleotides becomes rate-limiting for DNA synthesis. We have tested this possibility and found that the uptake and equilibration of exogenous TdR into the TdR nucleotide pool of infected cells is not affected by cycloheximide (T. H. Mainprize, J. Kowalski, and W. P. Cheevers, manuscript in preparation). This result has also been obtained by R. Hand (personal communication). Thus we have concluded that a relative decrease in the specific activity of [$^3$H]TdR nucleotides is not a plausible explanation for the drug-induced decrease in the utilization of [$^3$H]TdR for DNA synthesis, but rather such suppression is the result of prerequisite inhibition of protein synthesis.

It was previously shown that polyoma DNA synthesis is inhibited by cycloheximide exclusively at the level of initiation of new rounds of genome replication (18). Thus, it appears that the initiation step in viral DNA replication requires the concurrent formation of protein(s). The results described here suggest the involvement of a stoichiometric protein requirement for initiation of viral DNA replication since this process cannot be maintained at a normal rate for more than a few minutes in the absence of protein synthesis.

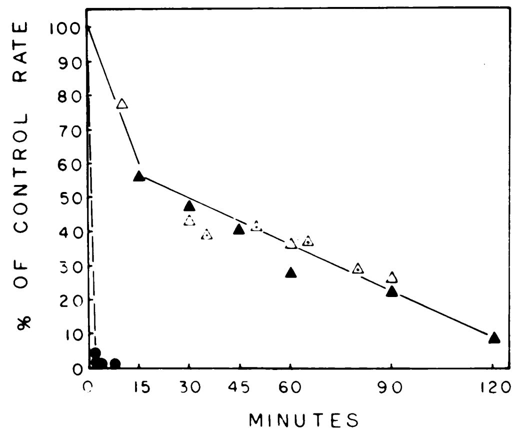


FIG. 2. *Kinetics of inhibition of protein and polyoma DNA synthesis by cycloheximide. The slope of cumulative incorporation curves in cycloheximide-treated cells for [$^3$H]amino acids (Fig. 1a) (●) and [$^3$H]TdR (Fig. 1b) (▲) are expressed as a function of the slope of incorporation curves in untreated cells. (Δ,◬) Additional experiments on viral DNA synthesis ([$^3$H]TdR incorporation curves not shown.)*

**Synthesis of form Ic viral DNA in cycloheximide-treated cells.** In the absence of protein synthesis, newly synthesized form I polyoma DNA is replaced by a viral DNA component referred to as form Ic (17). Form Ic is a monomeric closed-circular DNA species characterized by a two-thirds reduction in superhelicity as compared to form I. It is synthesized in the presence of cycloheximide on pre-existing form I templates without the intervention of progeny form I DNA as an intermediate; thus it arises by alteration of a protein synthesis-dependent step in the closure of the terminal intermediate of viral DNA replication. The purpose of the work described here was to measure the rate at which the synthesis of viral DNA is diverted from the formation of component I to component Ic.

Infected cells were pretreated for various periods of time with cycloheximide and incubated with [$^3$H]TdR to label replicating viral DNA. The viral DNA was separated from high-molecular-weight cellular DNA by sedimentation in neutral sucrose gradients and then resolved into components I and Ic by isopycnic centrifugation in cesium chloride-propidium diiodide gradients (4, 17). Results are shown in Fig. 4. As expected, all of the closed-circular viral DNA synthesized in the absence of cycloheximide exhibited the form I conformation (Fig. 4a). With increasing time of pretreatment with cycloheximide, however, the proportion of labeled DNA with the superhelix density of

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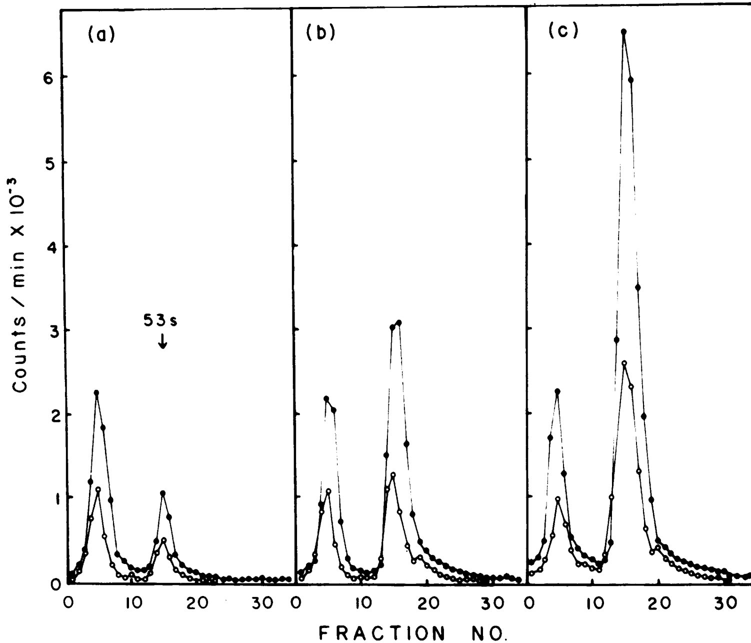


FIG. 3. *Alkaline sedimentation analysis of viral DNA synthesized in untreated and cycloheximide-treated polyoma-infected cells. Medium containing [³H]TdR supplemented with unlabeled TdR with or without cycloheximide (final concentrations of 25 μCi/ml, 0.1 μg/ml, and 10 μg/ml, respectively) was added to infected cultures at 24 h postinfection. At various times thereafter, viral DNA was isolated by neutral sucrose gradient sedimentation, denatured with NaOH, and centrifuged in alkaline sucrose gradients as described. (a) 15 min; (b) 30 min; (c) 60 min.* (●) *[³H]DNA, untreated cells;* (○) *[³H]DNA, cycloheximide-treated cells.*

form I progressively decreased; concomitantly, form Ic DNA, with a lower superhelix density, increased (Fig. 4b, c, and d).

The rate at which form I DNA was replaced by Ic in this experiment is shown in Fig. 5, expressed as a semi-logarithmic plot of the percentage of form I remaining after each pretreatment time. For comparison, the inhibition of [³H]TdR incorporation into total closed-circular DNA, measured by alkaline sedimentation and cesium chloride-propidium diiodide centrifugation, is also shown. It is clear from these results that in the absence of protein synthesis viral DNA replication is diverted from the formation of component I to component Ic with first-order exponential kinetics, whereas the rate of initiation of viral DNA synthesis is inhibited much faster and with different kinetics (see also Fig. 2).

From Fig. 5 it may be estimated that about 30 min in the presence of cycloheximide are required for one-half of newly synthesized DNA to acquire the superhelix density of form Ic. If this is correct, extrapolation of the decay curve predicts that form I DNA synthesis should essentially stop after 90 to 120 min in the absence of protein synthesis. This was confirmed by the experiment described in Fig. 6, in which the cumulative synthesis of forms I and Ic was followed in the presence of cycloheximide. Both species of closed-circular viral DNA were synthesized for approximately the first 90 min in cycloheximide-treated cells. Thereafter, essentially all of the new synthesized viral DNA was of the form Ic conformation.

We conclude from these findings and those of the accompanying paper (17) that, in the absence of protein synthesis, the synthesis of polyoma DNA is diverted from the formation of normal component I to component Ic according to the exponential decay of protein(s) involved in the closure of the terminal intermediate of viral DNA replication. The kinetics of formation of component Ic suggest that normal closure requires the synthesis of a single protein with an average lifetime of approximately 1.5 h.

## DISCUSSION

The present study and the two previous papers in these series (17, 18) have established that the control of polyoma DNA synthesis involves concurrent formation of protein minimally at two levels: (i) initiation of new rounds of genome replication, and (ii) closure of the

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terminal intermediate in viral DNA replication. On the basis of the kinetics of inhibition of these processes after interruption of protein synthesis by cycloheximide, it is suggested that the formation of at least two distinct proteins is required. The initiation of viral DNA replication requires essentially continuous protein synthesis, since this process cannot be maintained at a normal rate for more than approximately 10 min in the presence of cycloheximide. Closure, on the other hand, is much more stable after treatment of infected cells with cycloheximide. This process decays with first-order exponential kinetics, consistent with the involvement of a protein of average lifetime of approximately 1.5 h.

Within 15 min after the addition of cycloheximide the rate of synthesis of closed-circular polyoma DNA is reduced by approximately one-half via inhibition of the initiation step in viral DNA replication. After this initial rapid decay, viral DNA synthesis declines much more slowly, reaching a level of 10% of untreated cells only after about 2 h. This complex pattern of inhibition is not understood, although it may be a general feature of the relationship between protein synthesis and DNA replication. Weintraub and Holtzer (16) have obtained qualitatively similar results in eukaryotic cells treated with cycloheximide or puromycin. These authors termed this type of inhibition pattern of DNA synthesis "step-down." Similarly, Stan-

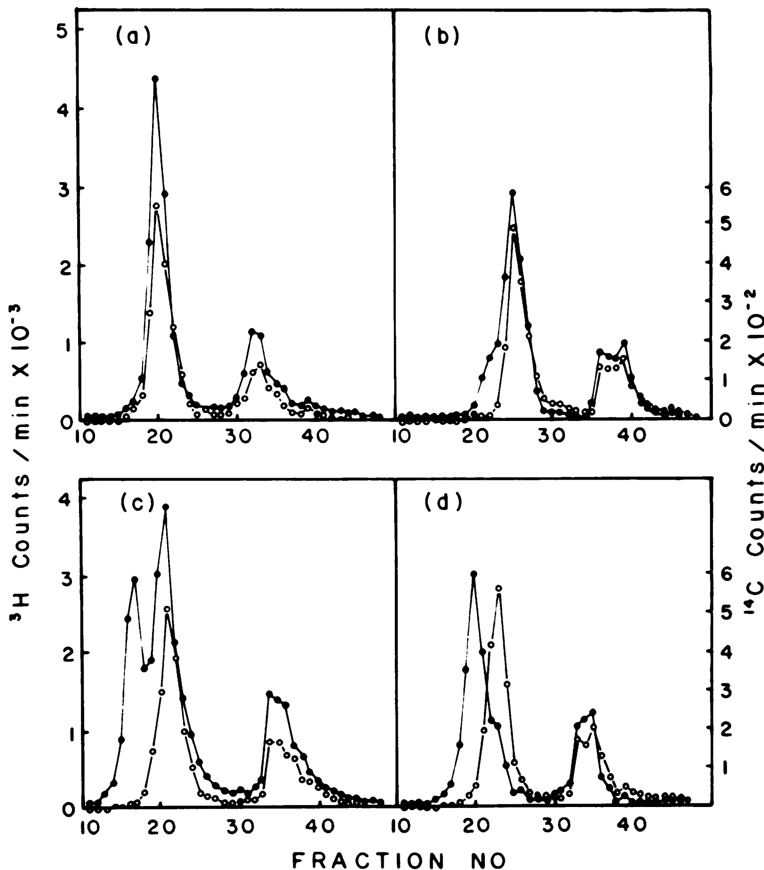


FIG. 4. *Isopycnic centrifugation in cesium chloride-propidium diiodide gradients of polyoma DNA synthesized in cycloheximide-treated cells. Infected cells were incubated with medium or medium containing cycloheximide for various times and labeled with [$^3$H]TdR (25 μCi/ml) at 27 to 28 h postinfection. Viral DNA was isolated by sedimentation in neutral sucrose gradients, and the buoyant density distribution of closed-circular components was then determined by isopycnic centrifugation in cesium chloride-propidium diiodide gradients (rotor SW50.1) (see references 4 and 17) (a) Untreated cells; (b) 10-min pretreatment with cycloheximide; (c) 30-min pretreatment with cycloheximide; (d) 60-min pretreatment with cycloheximide. (●) [$^3$H]DNA; (O) $^{14}$C-labeled form I viral DNA marker, isolated by neutral sedimentation analysis of DNA extracted from infected cells labeled with [$^{14}$C]TdR from 10 to 30 h postinfection (see reference 17).*

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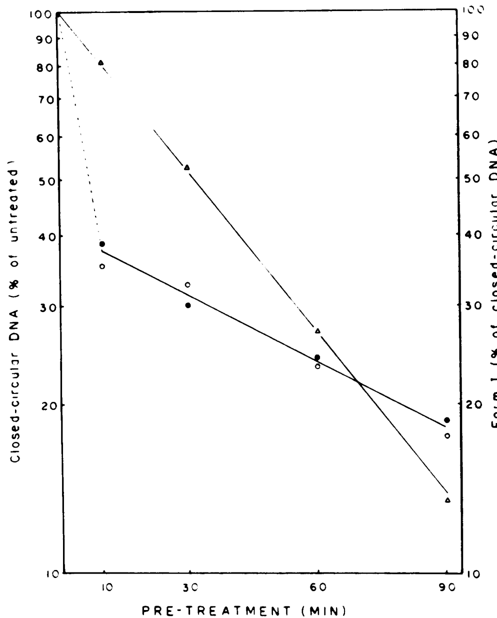


FIG. 5. *Rate of inhibition of form I DNA synthesis by cycloheximide. Infected cells were pretreated for various times with cycloheximide and labeled with [³H]TdR as described in Fig. 4. Viral DNA isolated by sedimentation in neutral sucrose gradients was concentrated by ethanol precipitation and dissolved in 0.3 M NaCl-0.001 M EDTA-0.01 M Tris, pH 8.1. An aliquot of each sample was denatured with NaOH, and total 53S closed-circular DNA was separated by velocity sedimentation in alkaline sucrose gradients (●). The remainder of each sample was analyzed by isopycnic centrifugation in cesium chloride-propidium diiodide (CsCl-PDI) to distinguish form I and form Ic components (buoyant density distributions for pretreatment times of 1, 10, 30 and 60 min are shown in Fig. 4).* (O) *Determination of total closed-circular DNA from CsCl-PDI gradients;* (Δ) *estimation of form I DNA from CsCl-PDI gradients.*

ners and Thompson (15) have shown that DNA synthesis is inhibited with step-down kinetics after interruption of protein synthesis in cells temperature sensitive for leucyl-transfer RNA synthetase. We have obtained the same results in exponential-phase mouse embryo cultures and for cellular DNA synthesis in polyoma-infected cells (Cheevers and Kowalski, unpublished data).

Weintraub and Holtzer (16) attributed the entire effect of the inhibition of protein synthesis on eukaryotic DNA replication to a reduction in the average rate of DNA strand elongation within replicons. This conclusion has been sustained in several other studies (7-9) but questioned by at least three groups (10-12). Our work (T. H. Mainprize and W. P. Cheevers, manuscript in preparation) indicates that cycloheximide does in fact reduce the rate of cellular DNA strand elongation, but this effect is secondary to an almost immediate block in the initiation of replicons. Thus, the primary effect of cycloheximide on both cellular and viral DNA replication is probably at the level of initiation.

From these and previous considerations on the temporal correlation of cellular and viral DNA synthesis in polyoma-infected cells (5, 6), we propose that the initiation of new rounds of viral DNA replication and the initiation of at least a portion of cellular DNA replicons are regulated by a common control mechanism, the elements of which include the continuous formation of an initiator protein. The possible role of integration of viral DNA sequences in this process is being investigated using temperature-sensitive mutants of polyoma virus defective in the initiation of viral DNA synthesis.

The involvement of protein in maintenance of the normal tertiary structure of form I polyoma DNA is not understood. It is possible that this protein functions as an inhibitor of other proteins which are known to remove superhelical turns from closed-circular DNA (3, 14). However, this mechanism cannot be simple compe-

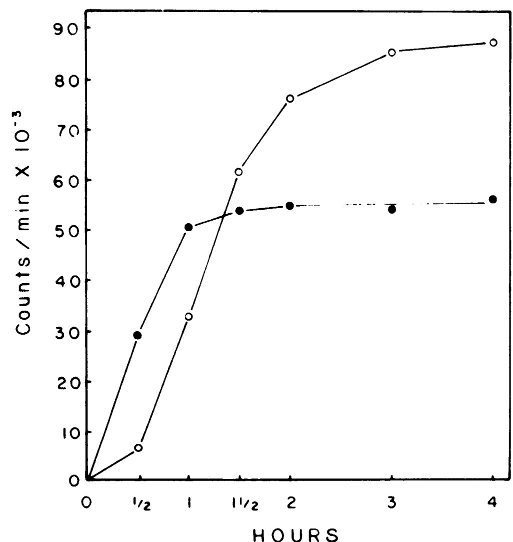


FIG. 6. *Accumulation of form I and form Ic viral DNA in cycloheximide-treated cells. Medium containing [³H]TdR (50 μCi/ml) supplemented with 0.1 μg of unlabeled TdR and cycloheximide per ml was added to infected cultures at 26 h postinfection. At the indicated times, closed-circular viral DNA was isolated and resolved into form I and Ic components by centrifugation in cesium chloride-propidium diiodide.* (●) *Form I DNA;* (O) *form Ic DNA.*

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tition between "untwisting" proteins and their inhibitors since the untwisting activity does not function to remove superhelical turns from newly synthesized form I viral DNA in the absence of protein synthesis (17). A more likely possibility is that untwisting proteins are not involved in maintenance of the superhelical structure of progeny DNA, but rather are enzymatic in nature and serve to create localized regions of unwinding during DNA replication (3, 14).

The model most closely consistent with present data is that the tertiary structure of viral DNA is maintained by a single protein with an average lifetime of approximately 1.5 h, which functions by attachment to newly synthesized DNA during or immediately after closure. This is suggested by the fact that the formation of component Ic requires prerequisite DNA replication, but upon reversal of inhibition of protein synthesis the Ic molecules may return to the normal superhelix density of form I by a process independent of replication; moreover, restoration of the form I conformation proceeds with the same kinetics as diversion of the synthesis of form I to Ic during cycloheximide inhibition (17). We are currently working on an in vitro assay for this protein based on the introduction of superhelical turns into purified component Ic.

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