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Topoisomerase I is preferentially associated with normal SV40 replicative intermediates, but is associated with both replicating and nonreplicating SV40 DNAs which are deficient in histones

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ABSTRACT

Based on the use of equilibrium centrifugation in CsCl to separate covalent complexes between topoisomerase I and DNA from protein-free DNA, it was concluded previously that the topoisomerase is preferentially associated with replicating SV40 DNA (Champoux, J. J. 1988. J. Virol. 62:3675–3683). One explanation for the failure to find the enzyme associated with nonreplicating viral DNA is that most of the completed DNA is rapidly sequestered for encapsidation and inaccessible to topoisomerase I. This explanation has been ruled out in the present work by the finding that topoisomerase I in COS-1 cells is also preferentially associated with the replicative form of an SV40 origin-containing plasmid that lacks the genes coding for the virion structural proteins and therefore cannot be encapsidated. Thus it appears that some structural feature of the replicating DNA or the replication complex specifically recruits the topoisomerase to the DNA. SV40 DNA which is produced in the presence of the protein synthesis inhibitor, puromycin, is deficient in histones and as a result lacks normal chromatin structure. Topoisomerase I was found to be associated with SV40 DNA under these conditions whether or not it was replicating. This observation is interpreted as an indication that under normal conditions, chromatin structure limits access of topoisomerase I to the nonreplicating viral DNA.

INTRODUCTION

Topoisomerases facilitate the interconversion of topological isomers of DNA by introducing transient single or double-strand breaks in the DNA (see references 1–4 for reviews). Type II topoisomerases act by passing an intact region of duplex DNA through a double-strand break in another region of the same or different molecule. Type I topoisomerases (topo I) act by introducing transient single-strand breaks into duplex DNA. Based on genetic studies, both the prokaryotic and eukaryotic type I enzymes appear to be dispensable for cell growth. However, under normal conditions they are probably responsible for providing swivels for both transcription (5–11) and DNA replication (12–16). Apparently, the type II topoisomerase can substitute for the type I enzyme in its absence.

Upon addition of protein denaturants to eukaryotic topo I reactions, a covalent complex is formed that links the enzyme to the 3′ phosphate at the site where the enzyme has nicked the DNA (17–20). Tabulation of nucleotide sequences in the vicinity of a large number of *in vitro* break sites revealed a consensus sequence for the breakage reaction. Covalent attachment preferentially occurs to T residues (−1 position) with either an A or T at the −2 position, a G or C at the −3 position and an A, G, or C at the −4 position (21). Sites that match this degenerate consensus occur with a high frequency in random sequence DNA and therefore this degree of specificity is unlikely to play a major role in limiting access of the enzyme to DNA *in vivo*.

The cytotoxic alkaloid, camptothecin (CTT), is a potent inhibitor of both RNA and DNA synthesis in eukaryotic cells (22). Since cells lacking topo I are unaffected by CTT, the type enzyme I must be the sole target of the drug (23–25). Although CTT has little or no effect on the relaxing activity of topo I *in vitro*, it markedly increases the amount of covalent enzyme-DNA intermediate that is detected when *in vitro* reactions are stopped with protein denaturants (26). The enhanced breakage appears to result from a retardation of the enzyme-mediated closure reaction by the drug (27, 28). The cytotoxic effects of the drug probably result from the formation of long-lived covalent adducts in duplex DNA *in vivo* that interfere with the propagation of replication forks and transcription complexes.

When SV40 DNA is isolated from infected monkey cells after lysis with sodium dodecyl sulfate (SDS) (29), covalent complexes between the viral DNA and topo I can be separated from protein-free DNA on the basis of their reduced buoyant densities in CsCl (13). Utilizing methodology based on this observation, we have shown that the amount of [^{3}H]-thymidine-labeled SV40 DNA found in the complexes is inversely proportional to the length of the labeling period and have concluded that topo I is preferentially associated with replicating SV40 *in vivo* (13). In addition, we have isolated the DNA-protein complexes and mapped to the nucleotide level the locations of topo I break sites

over approximately 30% of the viral genome (30). The sites were nonrandomly distributed on the DNA with no sites near the replication origin and a preponderance of sites on the strand that is the template for discontinuous DNA synthesis. This latter observation is not due to an intrinsic strand bias of topo I since *in vitro* studies revealed approximately equal numbers of sites on the two strands (21).

What is the basis for the observed preferential association of topo I with replicating SV40 molecules? We previously considered two alternative explanations (13). On one hand, it is possible that some structural feature of either replicating DNA or the replication complex is responsible for specifically targeting the enzyme to the DNA. Alternatively, the failure to find topo I associated with nonreplicating SV40 molecules could be due to the rapid entry of completed molecules into the pathway leading to encapsidation. If entry into the packaging pathway precludes access of topo I to the DNA, then the pool of accessible nonreplicating molecules could be relatively small. In the present study, it has proven possible to rule out the latter possibility by examining the association of topo I with SV40 origin-containing plasmids that are replicating in COS-1 cells (31) under conditions where no encapsidation can occur.

Does the specific targeting of topo I to replicating SV40 DNA require normal chromatin structure? It has previously been shown that SV40 replication continues in the presence of the protein synthesis inhibitor, puromycin, but after several cycles of replication, the newly-synthesized DNA is deficient in negative supercoils, presumably because the DNA is not packaged into the normal nucleosome structure after the histones become depleted (32). The results reported here reveal that topo I is associated with the nucleosome-free SV40 DNA whether or not it is replicating. This result indicates that under normal conditions chromatin structure is responsible for limiting access of topo I to non-replicating DNA.

MATERIALS AND METHODS

General methods

The procedures for infecting CV-1 cells with SV40, for labeling and extracting the viral DNA [method of Hirt (29)], and for analyzing the DNA by equilibrium centrifugation in CsCl or CsCl propidium diiodide have been previously described (13). Infected cells were labeled with [^{3}H]thymidine or treated with puromycin in medium lacking serum. Where indicated, the label was chased by replacing the labeling medium with medium containing 100 μg/ml thymidine and 10 μg/ml deoxycytidine.

Construction of plasmid pSVLT

The pBR322-derived portion of pSVL (Pharmacia), which lacks the poison sequences present in pBR322 (33), was used as a vector for cloning the origin (ori) and T-Ag coding sequences of SV40. pSVL was cut with *Sal*I and *Eco*RI and the ends filled in with Klenow. SV40 DNA was cut with *Nae*I and *Bam*HI and the *Bam*HI end was filled in with Klenow. The DNA fragments from the two digestions were mixed, ligated, and after transformation the resulting recombinant clones were screened for the desired plasmid containing the larger SV40 *Nae*I-*Bam*HI fragment. The orientation chosen for the experiments described here contained the SV40 *Nae*I site abutting the *Sal*I site from pSVL.

Transfection procedure

An adaptation of the method of Sussman and Milman (34) was used for the transfection of COS-1 cells with pSVLT DNA. The cells were grown until just subconfluent on plastic dishes (diameter 10 cm, Lux Scientific Corp.) in 10 ml of Dulbecco Modified Eagle medium (DME) supplemented with 10% calf serum (Hyclone). The cells were washed with 10 ml of DME without serum and exposed to 3.5 ml of a solution containing pSVLT DNA at 1 μg/ml and DEAE-dextran at 500 μg/ml for 4 hours at 37°C. The transfection solution was removed and the cells were shocked for 2 min at 20°C with 2 ml of 10% DMSO in Hepes buffered saline (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, and 21 mM Hepes, pH 7.1). The DMSO solution was removed and the cells rinsed with 5 ml of phosphate buffered saline, followed by the addition of 10 ml of DME containing 10% calf serum. At 28 h after the beginning of the transfection, the plasmid DNA was labeled and extracted as described previously for SV40 infected cells (13). The mock transfected plates were treated in the same manner except the DNA was omitted from the transfection solution. For transfection of cells grown on small plates (diameter 6 cm) the volumes of all the solutions were reduced by a factor of 2.

Hybridization analysis of CsCl gradient fractions

100 μl of each gradient fraction was diluted to 0.5 ml with 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA (TE). Sonicated salmon sperm DNA was added to a final concentration of 100 μg/ml and the samples were treated with proteinase K (30 μg/ml) at 37°C for 60 min. Two volumes of ethanol were added and the samples were left at 5°C for 16 h to precipitate the DNA. The samples were dissolved in 50 μl of TE and the DNA denatured by boiling. To each sample was added 50 μl of a solution containing 4×SSC (SSC is 15 mM sodium citrate, 0.15 M NaCl, pH 7.0) and 0.2% SDS. The labeled DNA in the samples was hybridized to 0.6 μg of denatured SV40 DNA on a 6 mm diameter nitrocellulose filter disk in a sealed tube at 65°C for 20 h. The radioactivity bound to the SV40 DNA-containing filter was corrected for the amount of label retained by a control filter included in each hybridization reaction.

Filter-binding assay for DNA-topo I complexes

The filter-binding assay was based on the procedure previously described by Coombs and Pearson (35). 10 μl of the Hirt supernatant obtained from transfected COS-1 cells that had been labeled for various lengths of time at 28 hours post transfection was diluted to 5 ml with TE containing 0.4 M NaCl. (The SDS in the sample interferes with the binding of the complexes at lower dilutions.) A GF/C glass fiber filter (Whatman) was washed with 2 ml of the same buffer on a vacuum manifold and the sample applied to the filter with moderate suction. The filter was rinsed with 5 ml of the same buffer, dried and counted using a liquid scintillation counter with a toluene-based scintillation fluid. To determine the total input label in each assay, the same volume of each sample was spotted onto a separate glass fiber filter and the DNA was precipitated on the filter with 5% trichloroacetic acid. The filter was rinsed with ethanol and counted as described above. For each labeling period, the values obtained for the totals and the filter bound DNA were corrected for the amount of label observed from a mock-transfected culture that had been labeled for the same length of time. In some cases the values for the mock transfected samples were equal to as much as 50% of the

values observed in the transfected samples. In spite of the rather large correction factor, the results from one determination to another agreed to within 10%.

RESULTS

Topo I is preferentially associated with replicating ori-containing plasmid DNA

SV40 DNA was extracted from infected CV-1 cells which had been labeled with [^{3}H]thymidine for 5 min in the presence of 10 μM CTT and the DNA was banded to equilibrium in neutral CsCl (Fig. 1A). We have previously shown that the shoulder on the low density side of the ^{3}H-labeled DNA peak that extends up to the top of the gradient contains SV40 DNA-topo I covalent complexes. When the cells were labeled for 60 min with the addition of CTT during the last 5 min of the labeling period, only a small amount of labeled material banded with a reduced buoyant density (Fig. 1B). These data confirm our previous results showing that after SV40 infection, the amount of labeled SV40 DNA associated with topo I is inversely proportional to the length of the labeling period (13).

To test whether encapsidation of the SV40 DNA was responsible for this observation, COS-1 cells that had been transfected with the ori-containing plasmid, pSVLT, were similarly labeled for either 10 or 90 min with the addition of CTT during the last 5 min of the labeling period. No encapsidation could occur in this case since the plasmid DNA lacked the late genes coding for the virion structural proteins. The labeled DNA was extracted with SDS as before and banded to equilibrium in neutral CsCl. Since transfection is much less efficient than infection, it was necessary to hybridize the labeled material in each gradient fraction to SV40 DNA to distinguish the plasmid DNA from the background of cellular fragments present in the samples. As can be seen in Fig. 2, substantially more low density material was apparent in the sample that had been labeled for 10 min (Fig. 2A) than in the sample labeled for 90 min (Fig. 2B). Prior treatment of the samples with

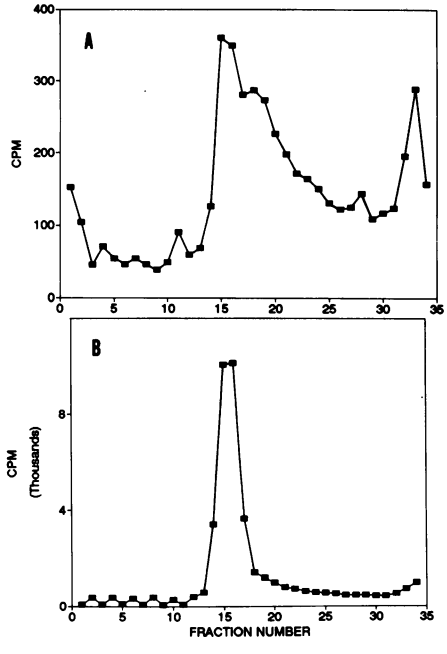

Fig. 1. Analysis of SV40 DNA-topo I complexes by equilibrium centrifugation in CsCl. At 42 h post-infection, SV40 infected cells were labeled with [^{3}H]thymidine for 5 min in the presence of 10 μM CTT (A), or for 60 min with the addition of CTT for the last 5 min of the labeling period (B), and the viral DNA extracted by the method of Hirt (29). The Hirt supernatant was banded to equilibrium in CsCl and individual fractions collected on filters and counted directly. The density in the gradient increased from right to left.

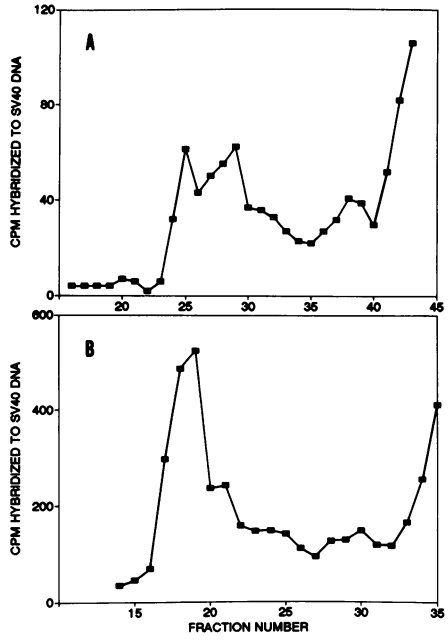

Fig. 2. Analysis of pSVLT DNA-topo I complexes by equilibrium centrifugation in CsCl. The plasmid pSVLT was transfected into COS-1 cells and at 28 h after transfection, the cells were labeled with [^{3}H]thymidine for 10 min (A) or 90 min (B). In both cases, CTT was added to 10 μM during the last 5 min of the labeling period. The DNA was extracted and banded to equilibrium in CsCl as described for Fig. 1. The radioactivity in the indicated fractions was hybridized to nitrocellulose filters containing SV40 DNA to determine the distribution of labeled plasmid DNA in the gradient. The gradient shown in A had 43 fractions while the gradient shown in B had 35 fractions.

Table 1. Association of topo I with pSVLT plasmid DNA

Labeling period min	Percent bound to glass filter[a] No CTT	+CTT	Percent replicative intermediates[b]
5	ND	ND	97
10	0	74	ND
20	5	65	73
40	0	51	53
60	7	41	39
90	3	25	ND

ND=Not done

[a]The total input label for each binding assay was determined by trichloroacetic acid precipitation of a portion of the Hirt supernatant on a glass fiber filter. The values obtained for mock transfected cultures were substracted from the values obtained for the totals and filter-bound complexes prior to calculating the percent bound to the filter for each labeling period. Repeat determinations agreed to within 10%.

[b]The percent of the label present in replicative intermediates for each labeling period was estimated as previously described (13) from the radioactivity that banded in a CsCl-propidium diiodide gradient at densities less than that of the closed circular DNA.

proteinase K eliminated the broad shoulder of material banding near the top of the gradient, (data not shown), confirming that the low density material resulted from the association of protein with the DNA. As expected, no low density material was observed if the CTT treatment was omitted (data not shown).

To circumvent the need to hybridize a large number of gradient fractions, the above experiment was repeated for a series of labeling times using an assay that depends on the binding of SV40 DNA-topo I complexes to a glass fiber filter (35). The results presented in Table I show that the percentage of the labeled plasmid DNA bound to the filter was indeed inversely proportional to the length of the labeling period. The failure to observe material binding to the filter in the samples which had not been treated with CTT indicated that topo I is the protein responsible for the filter binding. The percentage of the labeled DNA composed of replicative intermediates in each case was determined as before by measuring the amount of the labeled DNA that banded at positions less dense than closed circular form I DNA in a CsCl-propidium diiodide gradient (13). These values are also shown in Table I and correlate well with the percentage of the DNA bound to the glass filters for each labeling time. These results confirm the CsCl equilibrium centrifugation results and show that, as in the case with SV40 during a viral infection, topo I is preferentially associated with the replicating form of the DNA.

Topo I is associated with nonreplicating histone-deficient SV40 DNA

SV40-infected cells were exposed to the protein synthesis inhibitor, puromycin, for 30 min to deplete the cells of histones and allow several cycles of SV40 replication. The puromycin treatment was continued and the cells were labeled with [^{3}H]thymidine for either 10 min or 60 min with the addition of CTT to the labeling medium during the last 5 min of the labeling period. In both cases, inhibition of protein synthesis reduced the amount of incorporated [^{3}H]thymidine about 5-fold. The labeled viral DNA was extracted and banded to equilibrium in neutral CsCl (Fig. 3), and as expected, a substantial proportion ($\sim$80%) of the labeled DNA in the 10 min labeling period exhibited a reduced buoyant density due to bound topo I (13) (Fig 3A, closed boxes). In contrast with the results obtained in the absence of puromycin where only 21% of the DNA was found in the low density fractions of the gradient after a 60 min labeling period, approximately 70% of the labeled DNA produced in the presence of puromycin exhibited a reduced buoyant density (compare Figs. 3B and 1B). The association of topo I with the DNA in these experiments did not simply result from the inhibition of protein synthesis, since parallel control samples which had been treated with puromycin but not with CTT exhibited no low density material (Fig. 3A, x's; data not shown for 60 min labeling period). Thus, it appears that SV40 DNA that has finished replication in the presence of puromycin and is therefore deficient in histones is as accessible to topo I as replicating DNA.

Upon removal of puromycin, histone synthesis resumes and DNA which had been labeled in the presence of puromycin becomes supercoiled (32). Thus the DNA synthesized in the presence of the protein synthesis inhibitor can subsequently associate with histones. To test whether topo I is displaced from the DNA under these conditions, SV40 infected cells were labeled for 60 min in the presence of puromycin. One plate of cells was treated with CTT for 5 min and the DNA extracted as before, while a second plate was chased with unlabeled thymidine in the absence of puromycin for 30 min before the CTT treatment and extraction. After the chase, most of the low density material had disappeared (data not shown). Therefore reconstitution of SV40 chromatin within the cell without DNA replication (32) displaced topo I from the DNA.

The proportion of replicative intermediates is the same in the presence as in the absence of puromycin

Since we have shown previously that topo I is preferentially associated with replicating SV40 DNA (13), one explanation for the above result is that after puromycin treatment, a high proportion of the SV40 DNA that is labeled after exposure to [^{3}H]-thymidine for 60 min is in the act of replication, possibly by rapid reentry of newly synthesized molecules into the replicating pool. To test this possibility, SV40 DNA was extracted from infected cells that had been labeled with [^{3}H]thymidine for 60 min, either in the presence or in the absence of puromycin, and the DNA banded to equilibrium in a CsCl gradient containing the dye, propidium diiodide. In such a gradient, the dye-binding properties of the replicating molecules depend on the extent of replication and as a result, the molecules band at a spectrum of positions less dense than the closed circles (36–38). As can be seen in Fig. 4, the proportion of the labeled DNA comprising the replicative intermediates (the material less dense than the leading peak of closed circles) was the same in the presence of puromycin as in its absence (compare closed boxes in Figs. 4A and 4B), ruling out an effect of puromycin on the size of the pool of replicating molecules.

The results in Fig. 4A also confirmed that the DNA produced in the presence of puromycin lacked the normal number of

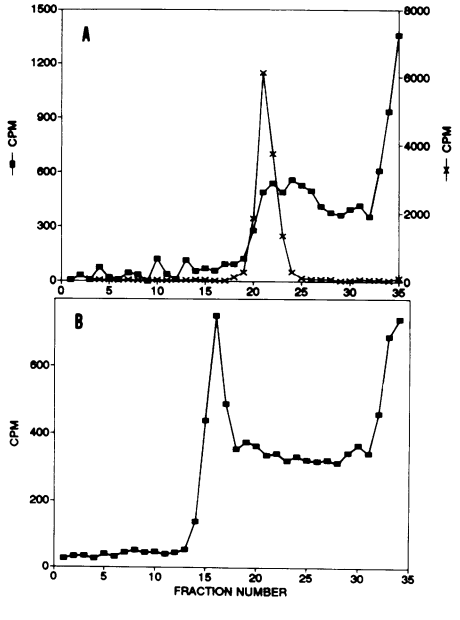

Fig. 3. Analysis of SV40 DNA-topo I complexes produced in the presence of puromycin by equilibrium centrifugation in CsCl. At 44 h post-infection, SV40 infected cells were treated with 0.4 mM puromycin for 30 min and then labeled with [^{3}H]thymidine in the presence of the same concentration of puromycin for 10 min (A) or 60 min (B). In both cases, CTT was added to the cultures to give a final concentration of 10 μM for the last 5 min of the labeling period. Radioactivity was determined by collecting individual fractions directly on filters (■). In a separate experiment, infected cells were treated with puromycin as above and labeled for 10 minutes with ^{3}H-thymidine in the presence of puromycin without the subsequent addition of CTT. Individual fractions were counted directly on filters and the results plotted on the graph shown in panel A (x).

negative supercoils and by inference lacked the normal nucleosomal structure. This conclusion follows from the fact that the DNA labeled in the presence of puromycin banded at a position approximately two fractions more dense than the negatively supercoiled ^{14}C-labeled marker SV40 (Fig. 4A, x's). It has been shown previously that this density difference reflects the difference between a normal SV40 DNA molecule containing ~25 negative supercoils and one containing few, if any, negative supercoils (39).

DISCUSSION

It has been demonstrated here that topo I is preferentially associated with a replicating SV40 origin-containing plasmid under conditions where no encapsidation of the DNA can occur. In addition, it has been shown that in the absence of histones, topo I is associated with nonreplicating SV40 DNA as well as with replicating molecules. For the discussion to follow, it is assumed that the observed distribution of topo I on SV40 DNA molecules reflects the sites where the enzyme had been functioning *in vivo* and that SV40 transcription complexes represent such a small proportion of the total SV40 DNA in the cell that they do not significantly contribute to the observed results (40). It is further assumed that the primary effect of puromycin treatment on SV40 replication is to exhaust the pool of histones in the infected cell (32).

Interpretation of the observation that topo I is associated with histone-deficient SV40 DNA even when it is not replicating depends on knowing the results of the experiments using the nonpackagable ori-containing plasmid (pSVLT). This follows from the fact that SV40 DNA that is replicated in the presence of puromycin and therefore lacks the normal chromatin structure is not encapsidated (32). Thus the finding that topo I is associated with nonreplicating SV40 in the presence of puromycin could be explained in principle, either by the failure of the DNA to be encapsidated or by the lack of histones on the DNA. Since topo I is not associated with nonpackagable pSVLT plasmid DNA when it is not replicating, it appears that it is the absence of histones on the DNA in the puromycin experiments that is responsible for the presence of excess topo I on the DNA. Therefore, the presence of normal chromatin structure appears to limit access of topo I to the DNA. In an analogous fashion, histones have been shown to block access of RNA polymerase to promoters (41). A recent report reaches a similar conclusion concerning the importance of chromatin structure in determining topoisomerase II sequence specificity *in vivo* (42).

Based on *in vitro* studies, one might expect that DNA with normal chromatin structure would be nearly as accessible to topo I as naked DNA. For instance, it has been shown that the DNA in SV40 chromatin is accessible to exogenously added topo I (43) and that breakage of the DNA in chromatin by added enzyme is only slightly reduced as compared to the breakage observed for naked DNA (44). These results are in sharp contrast to the results reported here where topo I is not found to be associated with nonreplicating SV40 chromatin. There are two possible hypotheses to explain this difference. SV40 chromatin might be altered in the process of isolating minichromosomes such that the DNA is more exposed and therefore accessible to topo I *in vitro*. Alternatively, chromatin structure may not completely block the binding of topo I, but the amount of topo I in the nucleus may be limiting such that the SV40 DNA (and cellular DNA) with normal chromatin structure is far from being saturated with enzyme. At the time after infection when the experiments reported here were carried out, cellular DNA synthesis had ceased, so only the newly replicated viral DNA lacked histones and presented itself as a preferred target for topo I. Consistent with this second hypothesis is our previous estimate that there is a maximum of one topo I molecule for every 8000 bp or ~1 1/2 SV40 equivalents of DNA (45).

What might be the basis for targeting topo I to replicating SV40 molecules? Three hypotheses are proposed. (i) Given the observation that nucleosome-free DNA is accessible to topo I, one possibility is that replicating DNA is deficient in histones. However, based on their sedimentation properties, SV40 replicative intermediates appear to possess a normal chromatin structure (46). Thus if such a mechanism acts to provide topo I access to the DNA, it is likely that only a short region of the replicating molecule is free of histones, possibly the region just in front of the advancing replication fork. (ii) Recent reports suggest that supercoiled DNA is a preferred substrate for topo I (47, 48). Therefore, it is possible that replication-generated positive supercoils are sufficient to recruit topo I to replicative intermediates. This mechanism could presumably function as well to recruit topo I to the positive and negative supercoils generated in actively transcribed regions of the genome (9). (iii) Alternatively, topo I may be physically associated with the replication machinery, thus ensuring that it is available to remove the supercoils as fast as they are generated by replication. If topo I were to be associated with some element of the replication machine that itself exhibits a strand bias during replication (DNA

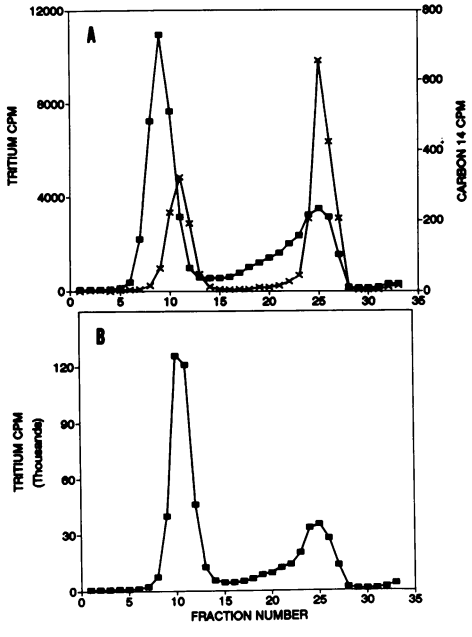

Fig. 4. Comparison of SV40 DNA made in the presence and absence of puromycin by equilibrium centrifugation in CsCl containing propidium diiodide. (A) SV40 infected cells were treated with 0.4 mM puromycin for 30 min and labeled with [^{3}H]-thymidine for 60 min in the presence of the same concentration of puromycin. The DNA was extracted as described for Fig. 1 and banded to equilibrium in a CsCl-propidium diiodide gradient (■). Prior to the gradient analysis, ^{14}C-labeled SV40 DNA was added to the sample to mark the buoyant position of normal supercoiled viral DNA (x). The ratio of nicked to closed circles in this preparation of ^{14}C-labeled SV40 DNA was approximately 2.(B) SV40 infected cells were treated as described for panel A except puromycin was omitted from the medium and no marker was included in the gradient.

polymerases or DNA helicases) then this explanation would account for the observed bias of topo I for the strand that is the template for discontinuous DNA synthesis.

In vitro systems have been developed for studying SV40 replication that employ naked DNA as the input template (49–51). Such systems properly initiate DNA replication in an origin-specific fashion and fork movement seems to mimic the process of elongation *in vivo*. However, the apparent requirement for normal chromatin structure for the proper association of topo I with replicating SV40 DNA *in vivo* suggests that such *in vitro* systems may not be useful for studying the involvement of topo I in DNA replication.

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