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Topological Complexity of SV40 Minichromosomes

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During attempts to measure the extent to which the proteins of simian virus 40 (SV40) minichromosomes restrain the ability of SV40 DNA to alter its twist in response to temperature changes, we found that temperature-shift-induced linking number changes are not reversible for isolated minichromosomes, suggesting that such changes, both in isolated minichromosomes and in cells, may be a consequence of structural alterations in chromatin proteins rather than of simple changes in DNA twist. We also found that the SV40 minichromosome pool is composed of subpopulations that display different responses to temperature shifts. For example, the linking number of DNA in newly replicated minichromosomes is more responsive to *in vivo* temperature changes than is the linking number of DNA in bulk minichromosomes. In addition, the linking number profiles of both isolated and intracellular minichromosomes change during the course of infection. These observations emphasize the topological complexity of SV40 minichromosomes and encourage caution in the interpretation of experiments carried out on bulk minichromosomes.

Keywords: simian virus 40; DNA topology; DNA topoisomerase; DNA replication; DNA flexibility

Introduction

DNA in eukaryotic cells exists in a complex with histones and other proteins called ''chromatin''. When chromatin is replicated, transcribed or folded, the DNA must change its ''twist'' (a measure of the rotational angle between adjacent base-pairs). It is therefore of interest to know the extent to which chromatin proteins hinder or facilitate alterations in DNA twist.

Studies of the effects of temperature shifts on the twist of closed circular plasmids in living cells of the budding yeast, *Saccharomyces cerevisiae* (Saavedra & Huberman, 1986; Morse *et al*., 1987), revealed that the DNA within these *in-vivo*-assembled minichromosomes is capable of about 70% of the alteration in twist exhibited by naked DNA. In contrast, previous experiments (Morse & Cantor, 1985) with nucleosomes reconstituted *in vitro* from chicken erythrocyte histones yielded results suggesting that vertebrate nucleosomal core particles completely prevent temperature-induced changes in twist, even within linker DNA. However, avian erythrocyte chromatin is inactive, synthesizing neither DNA nor RNA, whereas about 70% of yeast nuclear DNA is transcribed. Possible differences in composition and structure between *in-vivo*-assembled chromatin and *in-vitro*-reconstituted polynucleosomes must also be considered (Smirnov *et al*., 1991; Winzeler & Small, 1991). These issues can best be resolved by direct measurement of the effects of temperature shifts on the twist of *in-vivo*-assembled circular minichromosomes in vertebrate cells.

The simian virus 40 (SV40) minichromosome would seem to be an ideal model system for this purpose, since it is replicated, assembled into chromatin and transcribed using host-derived components, with the exception of the viral-specific transcription/replication factor, large T antigen. However, studies of SV40 minichromosome topology by several laboratories over the past decade have yet to provide definitive resolution of even basic issues such as the absence (Petryniak & Lutter, 1987; Lutter, 1989) or presence of unconstrained negative (Sundin & Varshavsky, 1979; Luchnik *et al*., 1982; Barsoum & Berg, 1985; Choder & Aloni, 1988) or positive (Ambrose *et al*., 1987; Esposito & Sinden,

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Abbreviations used: SV40, simian virus 40; NEM, *N*-ethylmaleimide; NP, nucleoprotein complex; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; MHL, modified Hirt lysis solution; PMSF, phenylmethylsulfonyl fluoride.

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1987) supercoils within the minichromosome. Similarly, there is no firm consensus regarding the extent to which SV40 DNA is restrained in its ability to change its twist in response to temperature variation. The change in twist observed by Lutter (1989) suggested that both linker and, to a small degree, nucleosome core DNA are free to alter their twist (are torsionally flexible). The findings of Ambrose *et al*. (1987) are consistent with the restriction of torsional flexibility to the linker regions alone, while Esposito & Sinden (1987) noted essentially no change in SV40 topology in response to temperature shifts.

Since variations in methodologies among these studies may account for the differences in reported topological properties of SV40 minichromosomes, we conducted a series of experiments to define the extent to which technical and biological variables affect minichromosome topology. We found that the most recently replicated SV40 minichromosomes as well as isolated minichromosomes in the NPI (Fernandez-Munoz *et al*., 1979) fraction are topologically distinct from corresponding bulk minichromosomes. Furthermore, topoisomer frequency profiles of SV40 minichromosomes vary with time post infection. Consistent with previous observations (Chen & Hsu, 1984; Esposito & Sinden, 1987; Chu & Hsu, 1992), these findings indicate that the bulk SV40 minichromosome population contains multiple components with distinct topological properties.

We also found that measurements of *in vivo* thermal unwinding of SV40 DNA are markedly affected by temperature-shift kinetics and the activity of endogenous topoisomerase during DNA recovery. Although we were able, under certain conditions, to detect reversible temperature-shift-induced changes in intracellular topoisomer frequencies, we could not confirm reversibility of comparable changes exhibited by isolated minichromosomes. Thus the proper interpretation of the topoisomer frequency shifts detected *in vivo* remains unclear.

Both the topological complexity of SV40 minichromosomes and the variable effects of growth and extraction conditions may account for the discrepancies among the results previously obtained by different laboratories investigating SV40 DNA topology.

Results

Temperature-shift experiments

Control of topoisomerase activity during cell lysis

Perhaps the most convenient, least intrusive means of measuring the extent to which chromosomal proteins hinder changes in DNA twist *in vivo* is to measure the change in linking number of a circular minichromosome in response to a temperature shift. Segments of DNA which are free to change their twist become more tightly twisted as the temperature drops (or less tightly twisted as the temperature rises). In the presence of active topoisomerase, these changes in twist are converted into changes in linking number (Morse & Cantor, 1985; Morse *et al*., 1987; Saavedra & Huberman, 1986; Shure *et al*., 1977; Petryniak & Lutter, 1987; Lutter, 1989; Ambrose *et al*., 1987; Esposito & Sinden, 1987).

So far this approach has yielded mixed results in the case of intracellular SV40 DNA. Ambrose *et al*. (1987) reported increases in average linking number of SV40 DNA when it was recovered from infected cells by Hirt (1967) extraction at $12\,(\pm 5)$°C compared to 40°C, suggesting to them that bulk intracellular SV40 minichromosomes contain some torsionally flexible DNA. However, other investigators (Shure *et al*., 1977; Esposito & Sinden, 1987) reported no significant changes in SV40 DNA topology over comparable temperature ranges, consistent with the conclusions of Morse & Cantor (1985; see Introduction). Thus this question merited further examination.

To distinguish between true *in vivo* topological shifts and those possibly resulting from the disruption of native conditions during cell lysis, it is crucial to prevent topoisomerase activity during all stages of DNA isolation. Endogenous topoisomerase activity has been previously observed during recovery of SV40 DNA by conventional Hirt extraction (Esposito & Sinden, 1987), so we included 11 mM *N*-ethylmaleimide (NEM) in the lysis buffer, since it is known to inhibit eukaryotic topoisomerases in detergent lysates over a broad temperature range (Saavedra & Huberman, 1986; Goto *et al*., 1984). To confirm the efficacy of NEM, we subjected SV40-infected CV-1 cells, cultured at 37°C, to Hirt extractions in the absence or presence of NEM at 0°C and 37°C approximately 24 hours after infection. This time after infection was chosen to minimize contribution to the topoisomer signal by mature virions (Blasquez *et al*., 1987; R.M.G. & J.A.H., unpublished observations). These samples were electrophoresed in parallel through a gel containing $75\ \mu g/ml$ chloroquine diphosphate. In such gels, closed-circular SV40 DNA molecules are displayed as a population of topoisomers, with each topoisomer differing from its neighbors by ± 1 in linking number. The directions of increasing linking number are indicated in Figure 1, which shows actual gel lanes, densitometer scans, and normalized topoisomer frequency profiles.

The 37°C samples had virtually identical topoisomer profiles regardless of NEM (Figure 1(a)) and despite a several-fold difference in the level of nicked DNA. However, extraction at 0°C in the absence of NEM resulted in an increase in linking number (Figure 1(b)) similar to that reported by Ambrose *et al*. (1987). Since NEM was present only during cell lysis, and since linking number changes require topoisomerase action, one can conclude from these observations that residual active topoisomerase must have been present during at least the

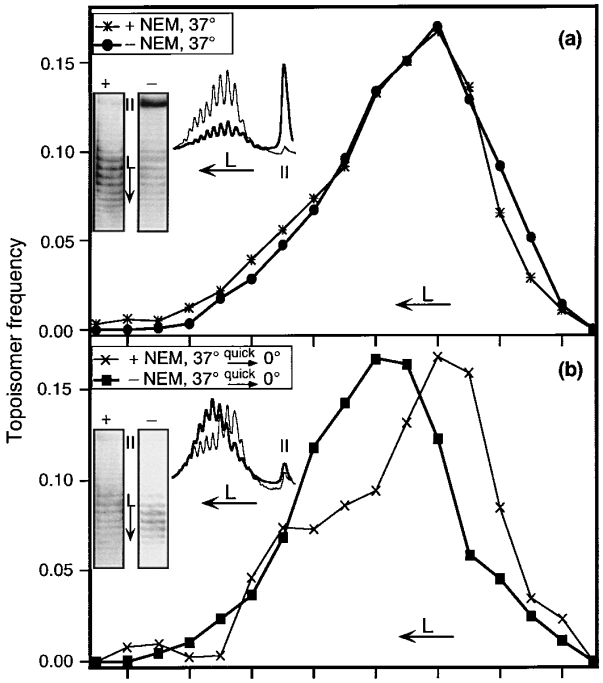

Figure 1. Effect of wash and lysis temperature on SV40 topoisomer profiles in the presence and absence of a topoisomerase inhibitor (NEM). In each topoisomer frequency profile the vertical axis indicates the proportion of the total SV40 supercoiled monomer signal represented by a given topoisomer. The horizontal axis corresponds to the relative linking number, starting on the left with the most highly linked (least negatively supercoiled) SV40 topoisomer visible in any lane of the gel depicted. The original gel lanes and tracings of the original densitometry data are shown as insets. Directions of increasing relative linking number (L) are indicated by the arrows. (a) Topoisomer frequency profiles resulting from wash and lysis at 37°C, 24 hours post infection, with (thin line) or without (thick line) 11 mM NEM in the lysis buffer (see Materials and Methods). (b) Topoisomer frequency profiles resulting from wash and lysis at 0°C, with (thin line) or without (thick line) 11 mM NEM in the lysis buffer. II, migration position of form II (nicked circular) SV40 DNA.

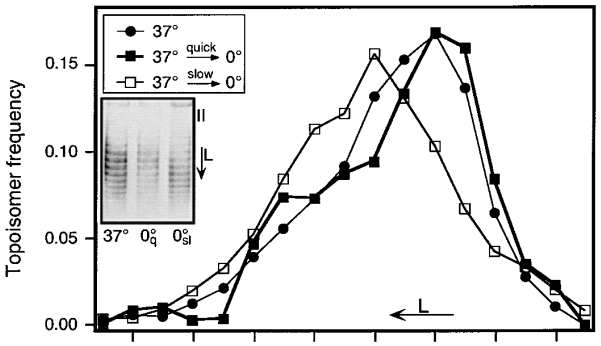

Figure 2. Effect of rate of *in vivo* cooling on SV40 topology. At a time 24 hours after infection, cells were lysed at 37°C (filled circles), at 0°C (filled squares), or were cooled slowly to 0°C (see text) prior to lysis at 0°C (open squares). NEM was included in all lysis buffers.

initial stage of cell lysis at 0°C in the absence of NEM. Thus, use of topoisomerase inhibitors during cell lysis aids in preserving the *in vivo* linking number.

Importance of gradual temperature shifts

To reliably measure linking number shifts in *S. cerevisiae* minichromosomes, it is necessary to shift the temperature gradually enough to permit endogenous topoisomerases to adjust the linking number to the new equilibrium value (Saavedra & Huberman, 1986). It is possible that the quick temperature shift from 37°C to 0°C employed in Figure 1 was too rapid to permit endogenous topoisomerase action prior to cell lysis, and that is why no linking number shift was observed unless NEM was absent from the lysis buffer.

To determine if longer cooling would result in a detectable linking number increase, a 24 hour post-infection culture was removed from the 37°C incubator, sealed and incubated sequentially for 30 minutes at 24°C, 30 minutes at 4°C, and 20 minutes at 0°C. The cells were then lysed at 0°C in the presence of NEM. The purified DNA was electrophoresed alongside parallel quick-cooled and 37°C samples already depicted in Figure 1. The resulting topoisomer profiles are compared in Figure 2. The profile of the slow-cooled sample is shifted to higher linking number despite the presence of NEM. These observations are consistent with the possibility that shifting the temperature from 37°C to 0°C leads to the introduction of unconstrained negative supercoils, and these supercoils are slowly relaxed by endogenous topoisomerases *in vivo*. This is supported by our finding that cultures subjected to gradual *in vivo* cooling followed by lysis at 0°C yield similar topoisomer profiles whether or not NEM is present during lysis (data not shown) in contrast to the marked NEM dependence of the profiles from rapidly chilled cultures (Figure 1(b)).

Reversibility

Since the linking number of SV40 DNA increases by two turns during virion assembly (Chen & Hsu, 1984; Ambrose *et al*., 1987), the redistribution of topoisomers observed when cultures are gradually cooled (as in Figure 2) may simply reflect encapsidation of minichromosomes or other irreversible change in chromatin structure during the prolonged temperature shift. If the redistribution is, instead, an indication of DNA torsional flexibility, it should be reversible.

The results of two experiments to test the reversibility of the stepwise cooling effect are presented in Figure 3. In each instance, two 24 hour post-infection cultures were gradually cooled to 0°C. One of these cultures was then lysed at 0°C. The other was returned to the 37°C incubator for

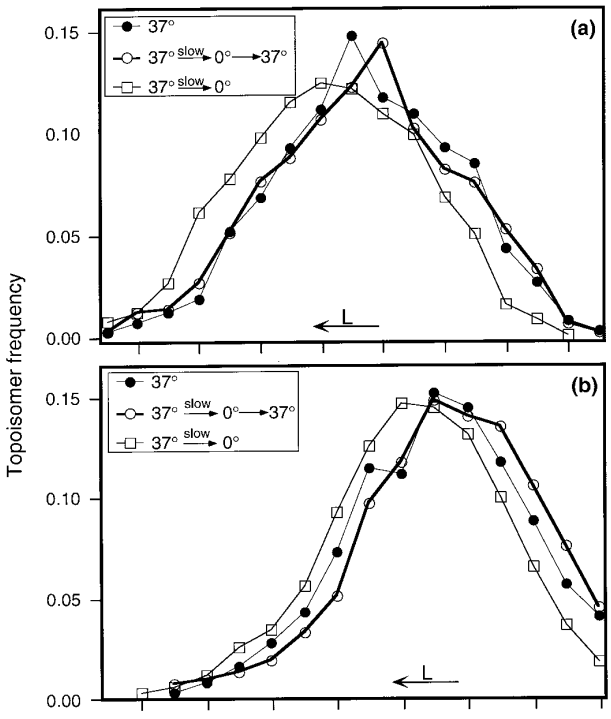

Figure 3. *In vivo* reversibility of cooling-induced shifts in SV40 topoisomer frequencies. The profiles shown were obtained in two independent series of experiments in which infected cells were cooled from 37°C to 0°C (a) over a 45-minutes period and held on ice for 60 minutes, or (b), over a five-hour period with two hours on ice, and then returned to 37°C for seven to eight minutes prior to lysis with 37°C buffers.

seven to eight minutes prior to lysis at 37°C. A third culture in each set was lysed directly at 37°C.

In both cases the return to 37°C seems to have reversed the shifts in topoisomer distribution associated with *in vivo* cooling (Figure 3). These results are consistent with the possibility that the effects of temperature shifts on the linking number frequency profiles of intracellular SV40 DNA reflect relaxation of supercoils induced by temperature-dependent variations in twist. However the apparent irreversibility of comparable linking number changes exhibited by isolated SV40 minichromosomes following *in vitro* temperature shifts, to be described later in reference to Figure 9, indicates that this interpretation may be incorrect.

Topological changes over time

The topological behavior of SV40 minichromosomes depends on factors such as chromatin composition and accessibility to topoisomerases. Such variables are expected to change as infection progresses. Chen & Hsu (1984) observed an increase in the average linking number of intracellular SV40 DNA between early (18 to 22 hours) and late (68 to 72 hours) stages of infection. We wished to determine if topological variations occur over shorter time intervals.

For this purpose, a series of parallel cultures grown at 37°C was lysed in the presence of NEM at four-hour intervals from 16 to 28 hours post infection (Figure 4(a) to (e)) and at 48 hours (Figure 4(a) and (f)) at 37°C or after quick cooling to 0°C as in Figure 1. The topoisomer profiles generated by the two different lysis protocols employed in Figure 4 are generally similar to each other at each time point after infection, confirming the results in Figures 1 and 2.

Between 16 and 28 hours post infection, there is a gradual decrease in the linking numbers of the modal SV40 topoisomers. At 48 hours post infection, the linking number distribution becomes more clearly heterogeneous, with a larger portion having a higher linking number, consistent with a contribution from mature virion DNA (known to have a higher linking number; Chen & Hsu, 1984; Ambrose *et al*., 1987).

Note that in Figures 1 and 2, asymmetric SV40 topoisomer profiles comparable to the 28 hour data shown in Figure 4(e) were obtained in an independent set of 24 hour post-infection extracts. Another independent set of 24 hour post-infection 37°C extracts, represented in Figure 3(a), yielded relatively symmetrical profiles more akin to the 20 hour results in Figure 4(c). These similarities suggest that minor variations in environmental conditions may lead to differences in the kinetics of infection between independent series of experiments such that profiles obtained at 24 hours post infection in one trial may resemble those at 20 or 28 hours in other trials.

These observations suggest that there is variation over time in the composition of the intracellular SV40 population with respect to topological properties. This variation may account for some of the discrepancies in topoisomer profiles obtained from independent infections. Thus, to isolate the effects of other variables such as temperature shifts on SV40 topology, it is best to make comparisons only among topoisomer distributions derived from parallel cultures.

Topological comparison of newly replicated and bulk DNA

It has been known for some time that newly replicated, or nascent, chromatin in mammalian cells differs markedly from bulk chromatin in several properties, including nuclease sensitivity and protein composition, before maturing within 10 to 20 minutes (reviewed by VanHolde, 1989). It was therefore of interest to examine the topological properties of the most recently replicated SV40 minichromosomes.

For this purpose, parallel 24 hours post-infection cultures were labeled with [^{3}H]thymidine at 37°C for seven minutes, then immediately subjected to lysis at 37°C or 0°C. This pulse length was selected because it is equivalent to about half the time required for a complete round of SV40 replication at 37°C (Perlman & Huberman, 1977). Thus, labeled

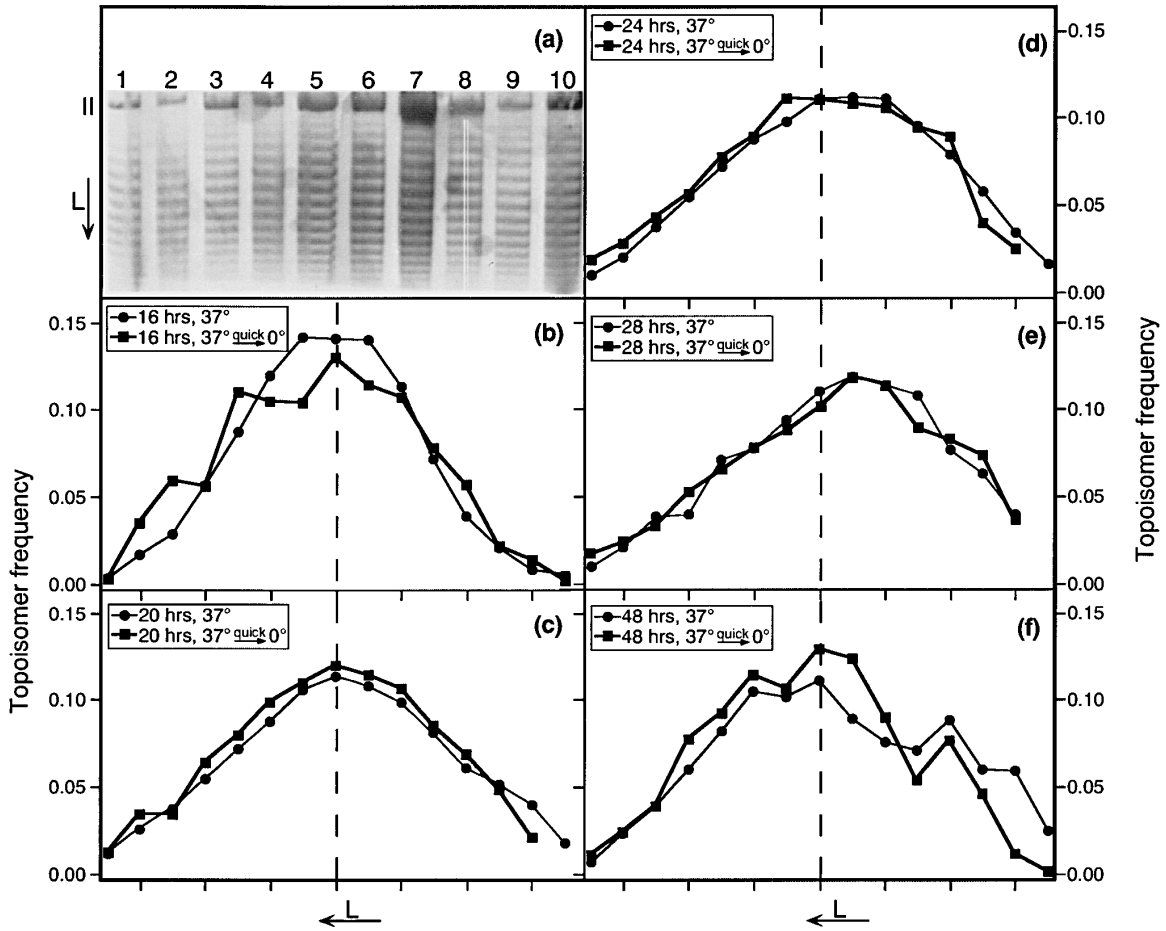

Figure 4. Variation of SV40 topology with time post infection. A series of parallel, SV40-infected cultures were lysed at 37°C (circles, odd-numbered gel lanes) or at 0°C (squares, even-numbered lanes) at the indicated times after infection. (b) Lanes 1 and 2; (c) lanes 3 and 4; (d) lanes 5 and 6; (e) lanes 7 and 8; and (f) lanes 9 and 10. The two white lines superimposed on gel lane 8 in panel (a) mark the densitometry scan path used to avoid the background spots (see Materials and Methods).

supercoiled SV40 monomers would represent nascent minichromosomes of only a few minutes in age.

To test whether prolonged incubation at 0°C following a quick shift from 37°C could produce reversible changes in bulk or nascent topoisomer distributions similar to those resulting from gradual cooling, three cultures in this series were pulse labeled at 37°C, then sealed and placed on ice following the addition of fresh, unlabeled, complete 0°C medium. After six hours on ice, one of these cultures was returned to 37°C for seven minutes prior to lysis with 37°C buffers. A second was subjected to lysis directly at 0°C. The third was left on ice overnight, then also lysed at 0°C.

The recovered DNA samples were electrophoresed and transferred to a nylon membrane, and the distribution of ^{3}H was determined by fluorography prior to hybridization with a (^{32}P)-labeled SV40 probe. The resulting autoradiogram and fluorogram are reproduced in Figure 5(a) and (b). Larger samples of the same DNA preparations were also analysed in an independent gel represented in Figure 5(c), in which linear (form III) SV40 DNA was resolved from the topoisomer ladder, thus permitting quantitation of linking number frequencies with minimal interference. In Figure 5, lane 1 contains a control sample from a culture in this series, which was lysed directly at 37°C without labeling.

Figure 6 presents a comparison of the ^{32}P and ^{3}H profiles in lanes 2 to 6 of Figure 5. The pulse-labeled topoisomers (thick lines) from cultures held at 0°C for six hours or overnight (Figure 6(c), (e), (h) and (j)) are shifted towards a higher linking number compared to controls maintained at 37°C (Figure 6(a) and (f)) or adjusted quickly to 0°C and then immediately lysed (Figure 6(b) and (g)). The similarity between the ^{3}H distributions in Figure 6(c), (e), (h) and (j) suggests that the pulse-labeled minichromosomes attained a topological steady state within six hours on ice. The shift of the pulse-labeled topoisomers towards higher linking number was reversed when a culture held at 0°C was returned to 37°C (Figure 6(d) and (i)). This reversible variation in topoisomer frequency profile with temperature suggests the possible presence of torsionally flexible DNA in a major portion of the

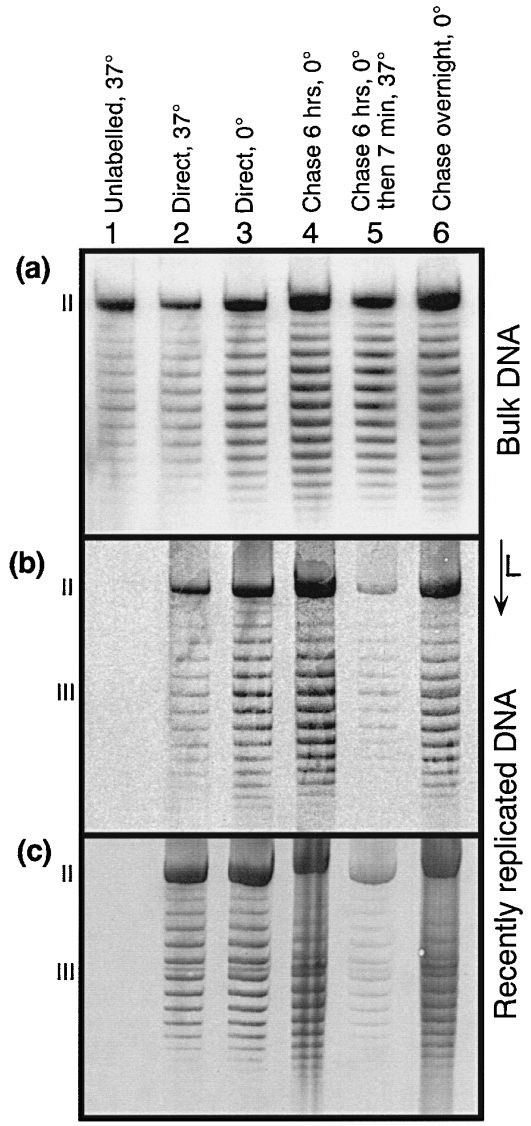

Figure 5. Comparison of bulk and recently replicated topoisomer profiles. (a) Autoradiogram of a Southern blot hybridized to a (^{32}P)-labelled SV40 probe. (b) Fluorogram of the same membrane prior to hybridization to the SV40 probe. (c) Fluorogram of a membrane from an independent gel with heavier loading of the same samples. Lane 1, cells directly lysed at 37°C. Lane 2, cells pulse-labelled with [^{3}H]thymidine at 37°C then lysed directly at 37°C. Lane 3, cells pulse-labelled at 37°C then directly lysed using 0°C buffers. Lane 4, cells pulse-labelled at 37°C then placed in fresh 0°C medium with serum on ice for six hours prior to lysis with 0°C buffers. Lane 5, same as lane 4 but returned to 37°C for seven minutes then lysed using 37°C buffers. Lane 6, same as lane 4 but held at 0°C overnight prior to lysis with 0°C buffers. Note; the culture represented in lane 5 was mistakenly placed on ice briefly before the pulse-labeling at 37°C and subsequent 0°C chase. This most likely accounts for its comparatively poor incorporation of [^{3}H]thymidine. II, migration position of form II SV40 DNA. III, migration position of form III (linear) molecules.

pulse-labeled minichromosomes. However, the caveat raised above regarding evidence of flexibility applies to this result as well. In contrast to the pulse-labeled DNA, topoisomer profiles from the bulk minichromosomes in this experiment were not appreciably shifted (thin lines in Figure 6(a) to (e)), perhaps because the *in vivo* relaxation rate of the bulk population may be extremely slow at 0°C and the cells were cooled too quickly to permit topoisomerase action at intermediate temperatures. Overnight incubation at 0°C did, in fact, result in a broadening of the bulk profile toward higher linking numbers (Figure 6(e)), and the bulk profile from a parallel culture subjected to slow *in vivo* cooling showed a modest reversible shift (Figure 3(b)).

Topological properties of isolated minichromosomes

The isolation and fractionation procedures described by Fernandez-Munoz *et al*. (1979) allow resolution of SV40 minichromosomes into two major populations with distinct properties. These were termed nucleoprotein complex I, or NPI, sedimenting around 70 S, and NPII, sedimenting more heterogeneously (100 to 200 S). The results of pulse-chase experiments proved the latter to be derived *in vivo* from the former (Fernandez-Munoz *et al*., 1979). Transcription and DNA synthesis were detected only in the NPI population, with NPII presumed to represent virion assembly intermediates. NPI DNA was subsequently found to have a lower average linking number than combined NPII and virion-derived DNA (Chen & Hsu, 1984).

To determine whether these physical and metabolic differences are reflected in topological properties, NPI and NPII were recovered from a matched set of cultures at 24 and 48 hours post infection, then subjected to temperature shifts in the presence of exogenous type I topoisomerase, followed by purification and analysis of their DNA. Bulk intracellular SV40 DNA was also collected at each time point by 37°C Hirt lysis in the presence of NEM.

Electron microscopic examination of material from an earlier preparation at 24 hours post infection (Figure 7) supports identification of the 70 to 80 S peak and the faster sedimenting material, respectively, as NPI and NPII (Fernandez-Munoz *et al*., 1979). Figure 7(a) to (c) show examples of what appear to be highly twisted nucleoprotein complexes visible in the NPI fraction. The objects seen in the NPII fraction (Figure 7(d) to (g)) consist of what seem to be lengths of tightly coiled nucleoprotein extending from dense, virion-like bodies, suggesting encapsidation intermediates (Blasquez *et al*., 1983). The bottom-most gradient fractions (Figures 7(h) & (i)) contained numerous bodies morphologically consistent with mature virions (Blasquez *et al*., 1983).

Electrophoretic analysis was performed on 24- and 48-hour samples in parallel gels, thus allowing direct comparison of topoisomer distributions from

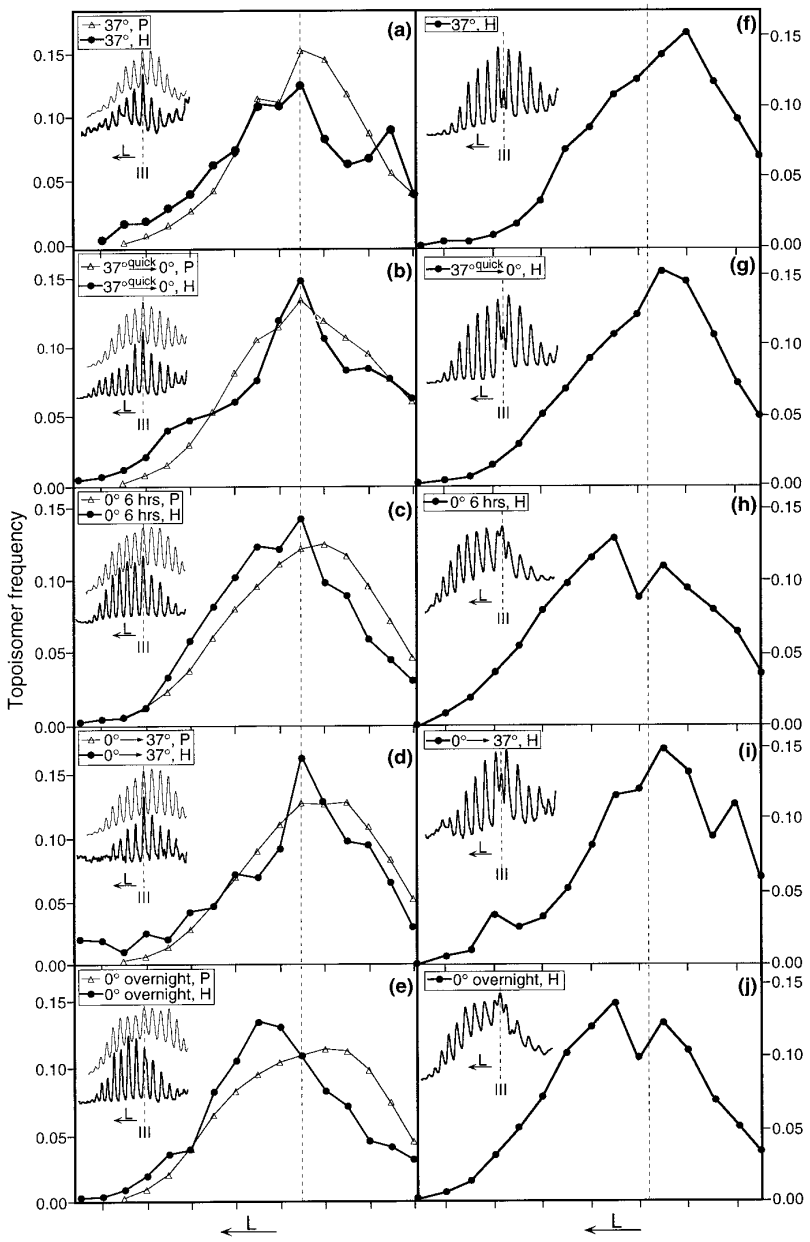

Figure 6. Comparison of bulk and pulse-labelled SV40 topoisomer frequency profiles. Panels (a) to (e) depict topoisomers resolved in gel lanes 2 to 6 of Figure 5(a) and (b). P, bulk topoisomers hybridizing to the [^{32}P]SV40 probe (see Figure 5(a)); H, [^{3}H]thymidine pulse-labelled SV40 topoisomers (see Figure 5(b)). Note; the broken lines in final plots in (a) to (e) indicate the overlapping migration positions of form III DNA and a form I topoisomer. Panels (f) to (j) depict data from gel lanes 2 to 6 of Figure 5(c), in which the form III position (indicated by the broken line) is resolved from the topoisomer bands.

the two time points. An autoradiogram representing the 48-hour samples is shown in Figure 8, and topoisomer frequency profiles of all samples are presented in Figure 9. The profiles for bulk DNA (isolated at 37°C) at 24 and 48 hours post infection differed from each other as did the profiles of the corresponding NPI fractions (topoisomerase-treated at 37°C; Figure 9(a) and (b)). At both times, however, the profiles of NPI samples (relaxed at 37°C) are shifted toward lower linking number values than those of bulk DNA (isolated at 37°C; Figure 9(a) and (b)). This is especially evident at 48 hours post infection, consistent with the findings of Chen & Hsu (1984).

Warming NPI samples to 37°C from the isolation and storage temperature of 0°C in the presence of exogenous topoisomerase resulted in a decrease in linking number (increase in negative supercoiling) at both 24 and 48 hours post infection (Figure 9(c) and (d)). The failure of a step-wise return to 0°C to restore the initial linking number distribution after the 37°C incubation is not due to exhaustion of the exogenous topoisomerase, as shown by control experiments in which several-fold higher amounts of naked SV40 DNA were used as substrate in the same buffer at comparable concentrations of enzyme (data not shown).

The apparent irreversibility of the warming-induced linking number decreases exhibited by NPI *in vitro* technically disqualifies these topological shifts as evidence for the presence of torsionally flexible DNA. This finding also suggests that the temperature-dependent linking number shifts observed *in vivo* may involve alteration of chromatin

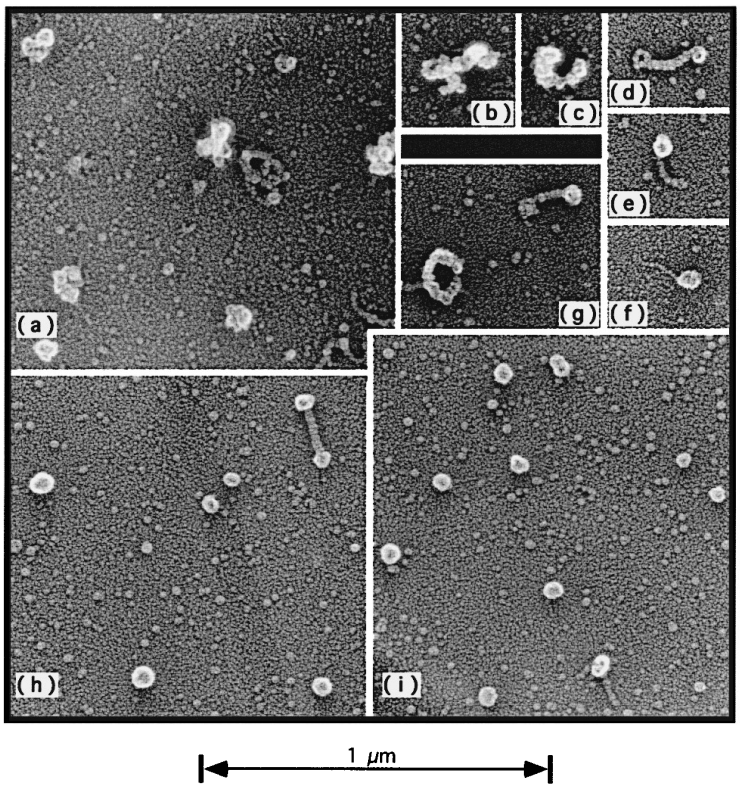

Figure 7. Presumed SV40 minichromosomes extracted at 24 hours post infection from partially broken cells in isotonic buffer with nonionic detergent (see Materials and Methods). Panels (a) to (c), presumed minichromosomes sedimenting at about 70 S, along with a peak of SV40 form I monomer DNA and ribosomal RNAs. Panels (d) to (g), presumed minichromosomes sedimenting at 100 to 180 S, along with a faster-sedimenting shoulder of SV40 form I DNA and little or no ribosomal RNA. Panels (h) to (i), presumed SV40 virus particles sedimenting at about 200 S along with a peak of both monomer and dimer SV40 DNA.

structure instead of passive DNA torsional responses to temperature changes. The putative chromatin reconfiguration appears to be readily reversible *in vivo* (Figure 3), but one or more components essential for the reverse reaction is evidently lacking *in vitro*.

We also checked for the presence of unconstrained superhelicity (torsional stress) in the NPI minichromosomes at 0°C by comparing linking number profiles before and after incubation with topoisomerase. Although control experiments confirmed the presence of active topoisomerase at 0°C, no significant differences in linking number profiles could be detected (Figure 9(e) and (f)). Thus there was no detectable unconstrained superhelicity in our NPI minichromosomes.

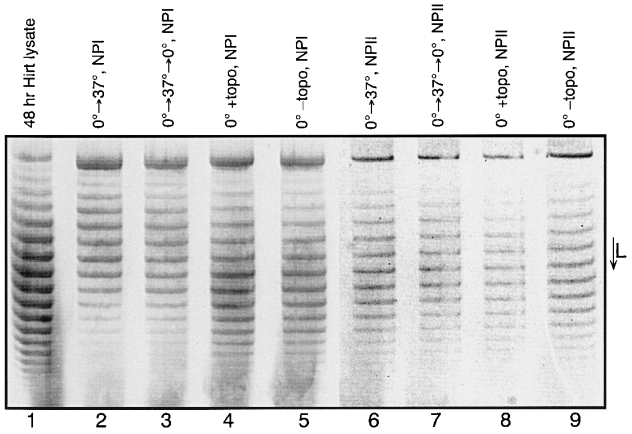

Figure 8. Resolution of SV40 topoisomers recovered from fractions NPI and NPII of 48 hours post-infection cultures. Samples were subjected to the indicated *in vitro* temperature-shift / topoisomerase treatments then analysed in parallel gel lanes. The portion of the autoradiogram containing lanes 6 to 9 (NPII) has been digitally intensified to facilitate visual comparison to the much stronger NPI and bulk signals.

Insufficient NPII DNA was recovered at 24 hours post infection to permit reliable topoisomer quantitation. At 48 hours post infection, the linking number distribution of NPII DNA (Figure 9(b)) closely resembled that of bulk DNA, in contrast to the linking number distribution of NPI. Effects of temperature shifts on NPII DNA (Figure 8) were far less pronounced than those on NPI DNA (Figures 8 and 9(c) and (d)) suggesting that loss of topological responsiveness to temperature precedes actual encapsidation, perhaps due to binding of the virion assembly protein VP1 to the minichromosomes (Blasquez *et al.*, 1986).

Discussion

DNA flexibility or chromatin reconfiguration?

We initiated these experiments in order to determine the extent to which chromatin proteins limit the ability of SV40 DNA to change its twist in response to temperature shifts. For these experiments we took advantage of the ability of topoisomerases to convert changes in twist into changes in linking number. We were not able to obtain a clear answer regarding the effects of

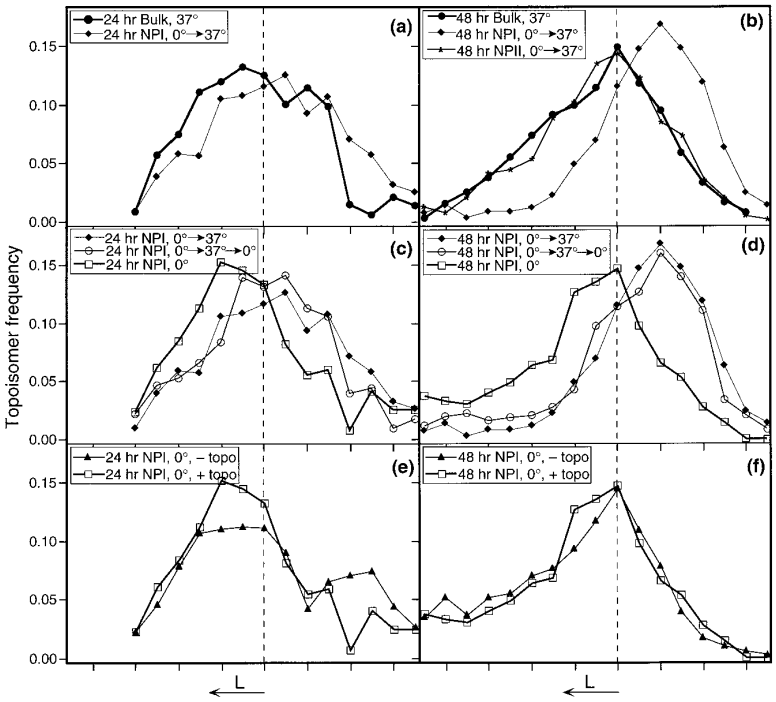

Figure 9. Topological properties of partially purified SV40 minichromosomes prepared at 24 ((a), (c) and (e)) or 48 ((b), (d) and (f)) hours post-infection. Panels (a) and (b), bulk SV40 DNA was recovered by our standard lysis procedure at 37°C (filled circles); NPI minichromosomes (70 to 80 S) were prepared at 0°C then incubated with topoisomerase I at 37°C before lysis with SDS and NEM at 37°C (filled diamonds); NPII fraction, 48 hours post infection, treated with topoisomerase I at 37°C (asterisks). Panels (c) and (d), NPI minichromosomes prepared at 0°C, then equilibrated with topoisomerase I at 37° as in (a) (filled diamonds); another portion of minichromosomes was equilibrated at 37°C as for the filled diamonds, but then cooled to and equilibrated with topoisomerase I at 0° before lysis at 0°C (open circles); a third sample of minichromosomes was prepared at 0°C then equilibrated with topoisomerase I and lysed at the same temperature (open squares). Panels (e) and (f), NPI minichromosomes prepared at 0°C (filled triangles); minichromosomes prepared in the same way, but then incubated at 0°C with topoisomerase I, as in (c) and (d) (open squares).

chromatin proteins on SV40 DNA twist, but our results do shed some light on the problem. First, we found that slow (∼1 hour) cooling from 37°C to 0°C yielded reversible *in vivo* modal linking number increases of one to two turns (Figures 2, 3 and 6). These values are much smaller than the value previously obtained for the similarly sized yeast 2 μm minichromosome (about five turns; Saavedra & Huberman, 1986). Thus the ''torsional flexibility'' of DNA in SV40 minichromosomes is certainly less than in yeast minichromosomes.

In fact, the torsional flexibility of SV40 minichromosome DNA may be significantly less than suggested by these small linking number changes. Although these linking number changes were reversible *in vivo* (Figures 3 and 6), they were not reversible *in vitro* (Figures 8 and 9). Linking number changes due to the effects of temperature shifts on torsionally flexible DNA should be reversible. Therefore the linking number changes that we have detected may not be due to torsional flexibility at all. Instead, they may be a consequence of temperature-induced reconfiguration of chromatin proteins. The *in vivo* reversibility of this reconfiguration (Figure 3) might have a cofactor and/or energy requirement that was not satisfied *in vitro*. Previous investigators have not directly tested the *in vitro* reversibility of the temperature-shift-induced minichromosome linking number changes that they have detected (Saavedra & Huberman, 1986; Ambrose *et al*., 1987; Lutter, 1989). Therefore, it is possible that these linking number shifts, previously attributed to DNA torsional flexibility, may instead be a consequence of chromatin structural alterations. In the case of yeast plasmid minichromosomes, however, temperature-shift-induced linking number changes do appear to be largely reversible *in vitro* (S. Y. Roth, personal communication).

Topological complexity

We observed marked differences between the topological properties of bulk SV40 minichromosomes and those of the newly replicated (Figures 5 and 6) and NPI (Figures 8 and 9) fractions. The linking number profiles of bulk minichromosomes (Figures 4 and 9) and of the NPI fraction (Figure 9) varied with the stage of infection, implying that the changes in bulk SV40 topoisomer frequencies as infection proceeds (Figure 4 and Figure 9(a) and (b); Ambrose *et al*., 1987; Chen & Hsu, 1984) reflect more than a simple accumulation of encapsidated molecules. In fact, minichromosomes at 16 and 20 hours post infection (Figure 4(b) and (c)), before any virion assembly, actually had higher linking numbers than minichromosomes at 24 and 28 hours post infection, suggesting that an increase in negative supercoiling may accompany the onset of expression of viral late genes.

Combined with the observations of Barsoum & Berg (1985), Chen & Hsu (1984), Esposito & Sinden (1987) and Chu & Hsu (1992), our findings support a view of SV40 minichromosomes as a dynamic composite of several topological pools differing in linking number profile and/or sensitivity to environmental conditions. This view helps to

explain why the topoisomer spread of SV40 minichromosomes is so much broader (Shure *et al*., 1977; Barsoum & Berg, 1985; Esposito & Sinden, 1987; Ambrose *et al*., 1987; this study) than would be predicted from their small content of torsionally flexible DNA (Shure *et al*., 1977; Ambrose *et al*., 1987; this study). Since the equilibrium spread of topoisomers about the mean linking number depends directly upon the available length of flexible DNA at the time of ring closure (Ambrose *et al*., 1987; Shure *et al*., 1977), the Gaussian topoisomer distribution for a functionally homogeneous sample of SV40 minichromosomes should be quite narrow, with only four or five species detectable by standard methods (Shure *et al*., 1977; Ambrose *et al*., 1987). The generally observed range is 15 to 20 topoisomers (Shure *et al*., 1977; Barsoum & Berg, 1985; Esposito & Sinden, 1987; Ambrose *et al*., 1987), indicating the presence of at least three topological subpopulations among intracellular SV40 nucleoproteins. Similarly, *in vivo* yeast plasmid topoisomer profiles have been observed which exceed their predicted equilibrium spread (Morse, 1991). Furthermore, the topoisomer profiles of these plasmids also change during the yeast cell cycle (Morse, 1991). Thus it seems that the presence of topological subpopulations may be a general feature of eukaryotic minichromosomes.

Although simple variation in number of nucleosomes per minichromosome could also explain anomalously broad linking number frequency distributions (Ambrose *et al*., 1987; Morse, 1991), some electron microscopic and nuclease digest measurements suggest that the actual heterogeneity in number of nucleosomes per minichromosome (Shure *et al*., 1977; Pederson *et al*., 1986; Lutter *et al*., 1992) is too small to fully account for the observed topoisomer spreads. Thus it seems likely that both functional complexity and nucleosome number heterogeneity contribute to the broad topoisomer distributions seen in eukaryotic minichromosomes.

One of the implications of population complexity is that the common practice of describing the topoisomer frequency profile of bulk SV40 minichromosomes with a single Gaussian curve is not justified. This approach assumes that the proportion of DNA molecules having a given linking number decreases exponentially with the square of the difference between that linking number and the statistical mean value for the sample (Shure *et al*., 1977; Ambrose *et al*., 1987). If more than one subpopulation is present, however, this condition is unlikely to be fulfilled, even if each of the subpopulations displays ideal Gaussian behavior.

A more acceptable means of describing such complex populations is the use of primary or normalized densitometric plots of electrophoretically resolved topoisomer ladders. With such plots, changes due to the behavior of subpopulations should be more readily detectable. Indeed, both our data (Figures 4, 6 and 9) and data from other investigators (Barsoum & Berg, 1985; Luchnik *et al*., 1985; Esposito & Sinden, 1987; Chen & Hsu, 1984; Choder & Aloni, 1988) provide examples of experiments represented by autoradiograms or densitometric plots in which the frequencies of topoisomers with certain linking numbers appear to change without significant effect on the mean linking number of the population as a whole. These effects would probably not have been apparent if the data had been described by single Gaussian curves.

The need for studies of more highly purified subpopulations

Although we attempted to isolate the behavior of specific subclasses of SV40 minichromosomes (newly replicated, those sedimenting at about 70 S, and those being packaged into virions), each of these operational subclasses had topoisomer spreads exceeding that of relaxed naked SV40 DNA (Shure *et al*., 1977; Ambrose *et al*., 1987), suggesting that it may comprise more than one topological pool. Thus, purification procedures capable of greater specificity are required. The necessary specificity might be achieved by using affinity adsorption techniques based on protein composition, presence of specific transcription or replication enzymes and factors, accessibility of certain DNA sequences (Morse *et al*., 1987) or the presence of specific transcripts. The problem of maintaining the native state of chromatin *in vitro* (Winzeler & Small, 1991; Smirnov *et al*.; 1991) can be avoided by performing topological manipulations *in vivo* prior to isolation and fractionation of the minichromosomes (Esposito & Sinden, 1987) in the presence of potent topoisomerase inhibitors. Analyses of the *in vivo* topological properties of such well-defined minichromosomes (Lutter, 1989) using standardized conditions may yield a clearer view than is now available of the relationships between topological properties and chromatin functions.

Materials and Methods

Cell culture

Monkey kidney (CV-1) cells were grown from frozen stocks in monolayer cultures using Dulbecco's modified Eagle medium (DMEM) supplemented with 8% (v/v) calf serum and 2% fetal calf serum (Life Technologies, Inc.) in 75 cm^2 tissue culture flasks. Temperature was maintained at 37°C and CO_2 at 5% (v/v). No antibiotics were used.

Infection with SV40

When cells reached approximately 90% confluence, the medium was decanted and the monolayer rinsed with prewarmed DMEM lacking serum. A 1:40 dilution of frozen SV40 stock (Gershey, 1980) in prewarmed DMEM was distributed among the flasks in sufficient volume (typically 2 to 3 ml per flask) to just cover the cells evenly and to provide about ten infectious virus particles per cell. The flasks were capped loosely and returned to the incubator for approximately two hours. During this time the flasks were occasionally rocked manually to ensure

uniform exposure. The viral suspension was then decanted, and fresh DMEM with serum was added to the cultures, which were returned to the incubator for the lengths of time indicated in the Results section.

Recovery of bulk intracellular SV40 DNA

After the *in vivo* temperature shifts described in the Results section, total intracellular SV40 DNA was recovered by a slight modification of the Hirt (1967) lysis procedure. After the medium was decanted, the cells were rinsed thoroughly but quickly with phosphate buffered saline (PBS: 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4) equilibrated to the desired lysis temperature. Immediately thereafter, 5 ml of modified Hirt lysis solution (MHL: 1% (w/v) SDS, 10 mM EDTA, 11 mM *N*-ethylmaleimide (NEM)) equilibrated to the desired temperature was added to the flask, covering the cells. The flask was held at constant temperature until lysis was judged complete (approximately seven to ten minutes at 37°C or 15 to 20 minutes at 0°C). Gentle swirling and rocking of the flask gathered the lysate into a gelatinous mass which readily decanted into a 50 ml centrifuge bottle without scraping. To remove bulk cellular DNA and debris, 1.25 ml of 5 M NaCl was mixed in by gentle inversion, and the lysate was incubated on ice for several hours prior to centrifugation at 25,000 ***g*** (15,000 rpm in a Sorval SS-34 (Du Pont) rotor) for 30 to 40 minutes. The cleared supernatant was sequentially extracted with phenol/chloroform/isoamyl alcohol (25:24:1) or buffered phenol (pH 7.3) then chloroform/isoamyl alcohol (24:1). The DNA was precipitated from the final aqueous phase by the addition of two volumes of absolute ethanol (EtOH) at room temperature. The precipitate was recovered by centrifugation at 13,000 ***g*** (11,000 rpm in an SS-34 rotor) for at least 30 minutes. The resulting pellet was rinsed with 70% (v/v) EtOH, then redissolved in TE-50:50 (50 mM Tris (pH 7.4), 50 mM EDTA). Excess RNA was removed by incubation of the samples for one hour at 37°C in the presence of DNase-free RNaseA at 10 to 20 units per ml. The sample was then made 2.5 M with respect to ammonium acetate and centrifuged (12,000 ***g*** for 10 minutes) to remove any precipitate. The clarified supernatants were transferred to fresh tubes. The DNA was recovered by EtOH precipitation as above, redissolved in TE-50:50 and stored at 4°C until used.

Electrophoretic resolution of topoisomers

Samples were subjected to electrophoresis in 1.2% (w/v) agarose gels containing 75 μg/ml chloroquine diphosphate (Sigma) according to the methods of Shure *et al*. (1977). Electrophoresis (40 to 45 volts) in vertical gels (15 cm long, 2 mm thick) was for 20 hours at 7 to 9°C.

DNA was transferred to nylon membranes (Zetabind, Cuno) and hybridized to a radioactively labeled SV40 probe (probe 5 of Nawotka & Huberman, 1988) as described by Nawotka & Huberman (1988).

Pulse labeling

Intracellular SV40 DNA was pulse labeled as previously described (Perlman & Huberman, 1977). Cells were rinsed with medium lacking serum at 37°C. An 8 ml volume of labeling solution ([^{3}H]thymidine, 50 Ci/mmol, 500 μCi/ml in DMEM without serum) pre-equilibrated at 37°C, 5% CO_2, was added to the culture, and incubation at 37°C was continued for seven minutes.

Fluorography

Prior to probe hybridization, membranes containing SV40 DNA from pulse labeling experiments were subjected to fluorography using EN^{3}HANCE spray (DuPont) as a scintillant according to the manufacturer's instructions.

Analysis of topoisomer distribution

Sample lanes on autoradiograms and fluorograms were scanned with a laser densitometer (LKB Ultroscan XL). Film exposures were selected which provided SV40 topoisomer peak signals of 0.2 to 2.0 absorbance units above background. Scan paths and widths were chosen that avoided any major irregularities in signal and/or background, as in Figure 4(a), lane 8. Data representing that lane were collected along a 1.6 mm path, marked by the two white lines in the gel picture, centered within a 4 mm spot-free corridor. In the case of Figure 5(b), lane 2, data from two separate scan paths were used to produce the composite ''spot free'' densitometry tracing shown in Figure 6(a).

Topoisomer signals were quantitated as follows. Densitometric scan peaks in positions matching the locations of visible SV40 topoisomer bands in the corresponding autoradiogram or fluorogram were separated by vertical lines extending from the base line through the points of minimum signal between identified band locations. These operationally defined peak areas were then cut out and weighed. The weights of all identifiable peaks were summed, and the fraction of the total weight represented by each was calculated and plotted as seen in the Figures. Note that modest errors in peak boundary assignments would only affect the relative values of the adjacent peaks and have no effect on the overall distribution of mass along the horizontal (linking number) axis.

Isolation of minichromosomes

Extraction of intact SV40 minichromosomes from cells at 24 and 48 hours post infection using the non-ionic detergent NP40 and their resolution by sedimentation through 5 to 40% (w/v) sucrose gradients were carried out in isotonic buffers by the procedure of Fernandez-Munoz *et al*. (1979) modified as follows. The extraction buffer included 2.5% sucrose along with 10 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin and 0.7 μg/ml pepstatin A as protease inhibitors and 11 mM NEM as a topoisomerase inhibitor. The three protease inhibitors were also present in the sucrose density gradient. The cells were chilled on ice prior to extraction and temperatures were kept below 4°C throughout the procedure.

Following centrifugation, the gradients were each divided into 25 to 28 fractions of 0.4 ml collected from the bottom of the tube. Samples of the fractions from each gradient were treated with 1% SDS at 65°C and analyzed in ethidium-bromide-containing agarose gels. At both time points, supercoiled SV40 DNA was detected in a broad peak cofractionating with rRNAs, presumably from 80 S ribosomes, with a shoulder that sedimented somewhat faster than rRNA. At 48 hours post infection the majority of SV40 DNA recovered, including all detected multimers, was found in the bottom-most gradient fractions, consistent with the sedimentation properties of assembled virions. No such material was

evident in the 24 hour post-infection preparation described here. Neither nicked circular nor linearized SV40 DNA was detected at this stage of analysis at either time point.

Gradient fractions comprising the NPI peak and the NPII shoulder were pooled separately and stored on ice.

Electron microscopy

Isolated SV40 minichromosomes (unfixed) were prepared for electron microscopy according to the methods of Fernandez-Munoz *et al*. (1979).

Topoisomerase treatment of isolated SV40 minichromosomes

Approximately three-quarters of each minichromosome pool were gently mixed with 33 units/ml calf thymus topoisomerase I (BRL) on ice. Portions of these mixtures corresponding to one half of the original pool volumes were transferred to a 37°C water bath and incubated for 45 minutes. At this point, half of each such sample was treated successively with NEM and then with 1% SDS (both for seven to ten minutes at 37°C), while the remainder was returned to 0°C following brief sequential incubations at room temperature (five minutes) and 4°C (15 minutes). All incubations at 0°C were terminated after two hour each by addition of NEM on ice followed ten minutes later by 1% SDS at 4°C. After addition of SDS all samples were heated briefly to 65°C to maximize dissociation of minichromosomes prior to DNA purification by organic extraction.

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