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Interaction of the DNA untwisting enzyme with the SV40 nucleoprotein complex

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ABSTRACT

The SV40 nucleoprotein complex which was isolated from infected CV-1 cells did not possess an active DNA untwisting enzyme. The superhelix density of the DNA in the chromatin complex was unchanged after treatment with purified rat liver DNA untwisting enzyme. However, in the presence of ethidium bromide (1 μg/ml) the superhelix density was changed. Moreover, the nicked intermediate in the DNA untwisting reaction could be detected using the chromatin DNA as a substrate. These results show that the DNA in the SV40 chromatin which is accessible to the DNA untwisting enzyme is under no topological strain.

INTRODUCTION

The simian virus 40 (SV40) nucleoprotein complex provides a convenient model system for studying eucaryotic chromatin. Infection of monkey cells with SV40 leads to the accumulation of the closed circular viral DNA in the form of a nucleoprotein complex with a sedimentation coefficient of 50-60 S (1,2,3). When isolated under physiological conditions (0.15-0.20 M NaCl), the viral DNA is associated with the five histones H1, H2A, H2B, H3, and H4 (4,5,6), as well as other nonhistone (7) and viral-specific proteins (7,8). In the electron microscope, the nucleoprotein complex appears as a circular fiber with the diameter of 100 Å which is characteristic of eucaryotic chromatin (9). The DNA is organized with histones into nucleosomes and, when examined in low salt, resembles a circle of "beads on a string" (6,9). Purified SV40 DNA contains negative superhelical turns (10); however, the topological state of the DNA in its native association with histone and non-histone proteins is unclear. We have been interested in determining whether any of the DNA in the native SV40 chromatin molecule is under topological strain.

The DNA untwisting enzyme provides a useful tool for the study of the topological state of the DNA within chromatin. Untwisting enzymes have been isolated from a variety of sources (11,12,13,14,15), and will remove super-

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helical turns from circular DNAs through the introduction of a transient nick (16). Any circular DNA containing either positive or negative superhelical turns will spontaneously convert to a relaxed state when treated with the untwisting enzyme.

Sen and Levine (17) have reported that SV40 nucleoprotein complexes purified through neutral sucrose gradients contained an associated untwisting activity. The SV40 chromatin-associated untwisting activity, when incubated with purified SV40 DNA, removed the superhelical turns present in the exogenous DNA, but left the DNA within the nucleoprotein complex unchanged in superhelix density. Similarly, Germond et al. (18,19) have shown that the superhelix density of the DNA in SV40 nucleoprotein complexes isolated either from disrupted virions or from infected monkey cells is unchanged following treatment with the DNA untwisting enzyme. There are two possible explanations for the resistance of the DNA in the nucleoprotein complex to relaxation by the untwisting enzyme: (i) the DNA in the nucleoprotein complex may be inaccessible to the untwisting enzyme, or (ii) the DNA in the complex which is accessible may exist in a relaxed configuration and remain unchanged in spite of repeated cycles of nicking and sealing.

A priori, one cannot assume that the untwisting enzyme should necessarily have free access to the DNA within chromatin. Much of the DNA is coiled with histones into nucleosomes, thus partially limiting the access of many enzymes to the DNA. Virtually all of the DNA within chromatin, for example, is accessible to DNase I, while a much smaller proportion of DNA is readily accessible to micrococcal nuclease (20). In addition, increasing evidence suggests that H1 histone and perhaps other nonhistone proteins may interact with the chromatin to condense and compact it (21). The compact chromatin DNA coated with protein might have very few, if any, accessible sites for the nicking-sealing activity of the untwisting enzyme.

From the reconstitution experiments of Germond et al. (18), one can infer that the DNA untwisting enzyme is active on SV40 DNA-histone complexes formed in vitro. However, there is no information available on the susceptibility of the DNA in complete chromatin particles (containing nonhistone and possibly viral proteins as well as histone proteins) to the untwisting enzyme.

We have re-examined viral nucleoprotein complexes for the presence of a DNA untwisting activity, and in addition, have studied the interaction between the complexes and highly purified DNA untwisting enzyme as a means for discriminating between these two alternatives. In this paper, we provide data which suggest that an active DNA untwisting enzyme is not associated with

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completed nucleoprotein molecules. Evidence is presented which indicates that purified DNA untwisting enzyme does have access to sites on the viral chromatin, but does not alter its superhelix density. This result suggests that the accessible DNA is not topologically constrained in the chromatin molecule as we isolate it.

MATERIALS AND METHODS

General. The sources for all reagents and materials have been given previously (22). BSC-1 cells obtained originally from E. Winocour, and CV-1 cells were grown in Dulbecco's modification of Eagle's medium (Grand Island Biological Supply Co.) supplemented with 10% fetal calf serum (North American Biologicals). A crude lysate of SV40 (strain 776 from N.I.H.) was used in these experiments. The procedures for the purification of SV40 DNA from infected BSC-1 cells have been previously described (22).

Enzymes. DNA untwisting enzyme was purified from rat liver nuclei as described previously (23). Proteinase K was obtained from Beckman, and was stored as a 1 mg/ml solution at 0°C. Protein concentrations were determined by the method of Lowry et al. (24).

Assay for the DNA untwisting enzyme. The DNA untwisting enzyme was quantitated with the DNA filter binding assay of Champoux and McConaughy (23). This assay makes use of the preferential binding of superhelical versus relaxed closed circular DNA to nitrocellulose filters at pH 11.1. Serial dilutions of an extract to be tested were incubated with a buffer containing 2 μg/ml of purified ^{3}H-labelled SV40 DNA (final KCl concentration 0.17 M) at 37°C for 10 min, stopped with 2 ml of assay buffer (0.39 M Na_3PO_4, 0.21 M NaH_2PO_4), and filtered through a nitrocellulose filter. The amount of enzyme which gave binding midway between substrate and product was defined as 1 unit of activity.

Isolation of viral nucleoprotein complexes. A modification of the procedures described by Green et al. (1) and Hall et al. (2) was used to isolate the viral nucleoprotein. Eight confluent large plates were infected at a multiplicity of 10 PFU/cell with SV40, adsorbed for 1 hr, and overlaid with medium. At 33 hr post infection, the medium was replaced with medium containing either 5 μCi/ml or 20 μCi/ml of [^{3}H]thymidine (20 Ci/mmole). The cells were harvested at 36 hr when the maximum rate of viral DNA synthesis was attained. The cells were lysed by the addition of 0.9 ml per plate of 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.25% Triton X-100. The plates were rocked gently for 10 min at room temperature, scraped into an ice cold centrifuge tube, and centrifuged at 500 X g (International PR-2 Centrifuge) for 10

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min at 0°C. After this step the complexes were kept between 0°C and 4°C. The supernatant was discarded, and the nuclei were resuspended in 2 ml of the same buffer containing 0.2 M NaCl. The nuclei were placed on ice for 3 hr with occasional mixing, followed by a high speed centrifugation to remove non-viral chromatin (Beckman Type 40, 23,000 rpm, 30 min, 4°C). The resulting supernatant (2 ml) was layered directly onto a 36 ml 5-20% sucrose gradient containing 10 mM Tris-HCl (pH 7.5), 0.15 M KCl, 1 mM EDTA, and centrifuged as described in the legend to Figure 1. The supernatant obtained from 8 plates of mock-infected cells was prepared in the same way and centrifuged in parallel. The gradients were collected in 1 ml fractions and an aliquot from each was spotted onto 3 MM paper filters, washed and counted for radioactivity as described previously (25).

Conditions for DNA untwisting enzyme treatment of SV40 nucleoprotein complexes. For untwisting enzyme treatment of the chromatin complexes in the presence or absence of ethidium bromide, SV40 nucleoprotein complexes (5 μg DNA) taken from Region III shown in Figure 1 were incubated with 0.5 μg of purified DNA untwisting enzyme for 30 min at 37°C in 10 mM Tris-HCl (pH 7.5), 0.15 M KCl, and 1 mM EDTA. Reaction mixes were stopped with 0.5% sarkosyl and analyzed in a CsCl-propidium diiodide gradient as described below. Detection of the nicked intermediate involved incubation of SV40 nucleoprotein complexes (0.15 μg DNA) with 0.68 μg of purified DNA untwisting enzyme for 10 min in 10 mM Tris-HCl (pH 7.5), 0.15 M KCl, and 1 mM EDTA at 23°C. In one vessel, the reaction was stopped with high pH (see below), while an identical reaction mix was incubated an additional 10 min at 23°C in the presence of 1 M NaCl before the high pH treatment.

CsCl-propidium diiodide equilibrium centrifugation. The reactions described above were stopped with sarkosyl (0.5%), extracted twice with phenol, twice with chloroform:isoamyl alcohol (25:1), and dialyzed extensively against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (TE). The final volume was adjusted to 1.45 ml with TE, and 1.40 g CsCl was added, followed by 0.25 ml of a 4 mg/ml solution of propidium diiodide. Centrifugation was carried out for 40 hr at 18°C and 35,000 rpm in the Beckman SW 56 rotor. Gradients were fractionated into approximately 40 fractions and radioactivity determined as described above.

Detection of the nicked intermediate by alkaline sucrose sedimentation. The reaction mixes described above were stopped by dilution to 0.15 mls with a mixture of NaOH and EDTA yielding a final concentration of 0.12 M NaOH and 10 mM EDTA. The reaction was then neutralized with 38 μl of 0.25 M Tris-HCl

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(pH 7.5) and 0.5 M HCl, and treated with proteinase K at a final concentration of 100 μg/ml for 30 min at 37°C to remove bound protein from the broken strands (26). The mixture was then brought to high pH with the addition of 5 μl of 10 M NaOH, and layered onto a 5-20% sucrose gradient containing 0.25 M NaOH, 0.75 M NaCl, 1 mM EDTA. The gradients were centrifuged in the Beckman SW 56 rotor for 80 min at 18°C and 55,000 rpm.

RESULTS

In an attempt to isolate the SV40 nucleoprotein complex in its most native configuration, the extraction was carried out under conditions which approximate physiological salt concentrations and pH.

SV40 infected CV-1 cells were labelled with [^{3}H]thymidine at 36 hr post infection. The nucleoprotein complex was leached out of isolated nuclei in a buffer containing the nonionic detergent Triton X-100 and 0.2 M NaCl at 0°C. Cellular chromatin was removed by centrifugation and the viral nucleoprotein isolated by neutral sucrose gradient sedimentation. A parallel gradient contained the corresponding material from mock-infected cells. Results of the centrifugation are shown in Figure 1. The viral nucleoprotein has an approximate sedimentation coefficient of 63 S as estimated from purified SV40 DNA (21 S) sedimented in a parallel gradient as a marker. Electron microscopy of material taken from Region III of Figure 1 using the method of Griffith (9) showed the compact 100 Å fiber characteristic of the native SV40 chromosome (data not shown).

Evidence that the DNA untwisting enzyme is not preferentially associated with the complex. The regions indicated in Figure 1 from gradients prepared with SV40-infected cells or mock-infected cells were pooled and immediately assayed for untwisting activity by the DNA filter binding assay, using an exogenous ^{3}H-labelled DNA as substrate (23). Quantitation of DNA untwisting activity in the pooled regions is shown in Table 1. Although detectable levels of untwisting activity were present near the top of gradients from either mock-infected or SV40-infected cells, no activity was detectable at a one to ten dilution of the pooled region containing the bulk of the complex DNA. A trace of untwisting activity was detected in Region III from both mock-infected and SV40-infected cells when undiluted pooled Region III was incubated with ^{14}C-labelled SV40 DNA for 30 min and analyzed in a CsCl-propidium diiodide gradient When the nuclear isolation step was omitted during the preparation of the viral chromatin, untwisting activity was found throughout the gradients from both mock-infected and SV40-infected cells with no quantitative differences between them. The general peak of activity in both of these crude preparations sedi-

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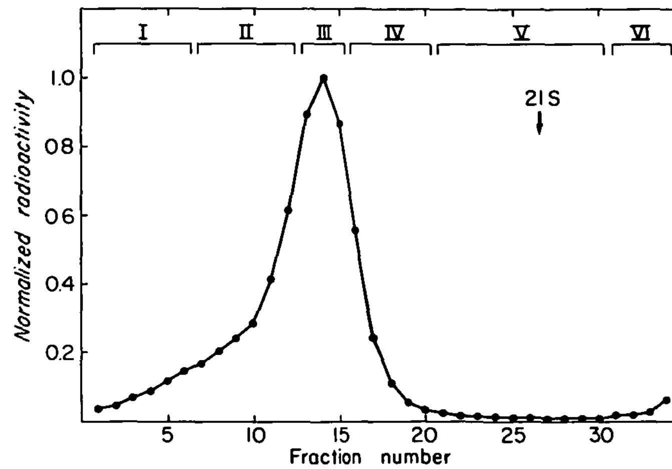

Figure 1: Neutral Sucrose Sedimentation. The supernatant obtained from nuclear preparations of mock-infected or SV40-infected cells was layered onto a 36 ml neutral sucrose gradient containing 10 mM Tris-HCl (pH 7.5), 0.15 M KCl, and 1 mM EDTA, and centrifuged 5 hr in a Beckman SW 27 rotor at 25,000 rpm and 4°C. Indicated regions were pooled and assayed immediately for DNA untwisting activity (see Table 1). The DNA concentration in Region III in a representative experiment was 50 μg/ml. The arrow indicates the position of purified SV40 DNA run as a marker in a parallel gradient.

TABLE 1

REGION	UNTWISTING ACTIVITY	
	SV40-Infected	Mock-Infected
I	<28*	<28*
II	<28*	<28*
III	<28*	<28*
IV	200	250
V	83	100
VI	238	208

The untwisting activity shown above represents a recovery (in total units layered on) of 31.9% for the gradient from mock infected cells and 39.2% for the gradient from SV40 infected cells. Region III for the gradient from SV40 infected cells had a DNA concentration of 52 μg/ml.

* These values represent the limits of detectability for the assay.

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mented slightly behind the nucleoprotein complex in the gradient, and may represent fragments of cellular chromatin containing bound enzyme. Mixing experiments with complexes from Region III and known amounts of DNA untwisting enzyme did not reveal the presence of any inhibitors in the nucleoprotein portion of the gradient. Isolation of the viral nucleoprotein by the method of Su and DePamphilis (27) also yielded a complex sedimenting at 60S with no detectable associated untwisting activity.

Effects of DNA untwisting enzyme on nucleoprotein complexes in the presence and absence of ethidium bromide. Viral chromatin complexes taken from Region III of Figure 1 were incubated with DNA untwisting enzyme for 30 min at 37°C in the absence of ethidium bromide, and the reaction was stopped with sarkosyl. Purified ^{14}C-labelled SV40 DNA was added as a marker, and the reaction mix was deproteinized, dialyzed and centrifuged to equilibrium in a CsCl-propidium diiodide gradient. Figure 2a shows that DNA untwisting enzyme had no effect on the superhelix density of the nucleoprotein complex DNA since the ^{3}H-labelled DNA was indistinguishable in buoyant density from the ^{14}C-labelled marker DNA. When purified ^{14}C-labelled SV40 DNA was mixed with the viral chromatin and similarly treated with untwisting enzyme, it shifted to the position of completely relaxed DNA (no superhelical turns) in the gradient (data not shown). This result confirms that the untwisting enzyme is indeed active in the presence of the nucleoprotein complex. These data agree with Sen and Levine's observation that the DNA untwisting enzyme has no effect on the superhelix density of the DNA in SV40 chromatin (17). Occasionally, a small portion of the nucleoprotein complex DNA (<10%) exhibited a higher buoyant density in CsCl-propidium diiodide after treatment with the untwisting enzyme. This observation probably reflects some heterogeneity in the SV40 nucleoprotein complexes. Heterogeneity may result from the *in vitro* isolation procedures employed, or it may represent molecules at different stages of maturation which contain variable amounts of histone and nonhistone proteins.

In order to test whether the failure to see a change in the superhelicity of the DNA in the complex was due to a relaxed state for the DNA, the isolated viral nucleoprotein was treated with untwisting enzyme in the presence of 1 μg/ml of ethidium bromide. At this ethidium bromide concentration, native SV40 DNA will take on a positively superhelical configuration (28) which can be altered by the nicking-sealing activity of the DNA untwisting enzyme (12). The DNA product of an untwisting reaction carried out in the presence of ethidium bromide contains more negative superhelical turns than native SV40 DNA when measured in the absence of dye, and thus will exhibit a lower buoyant

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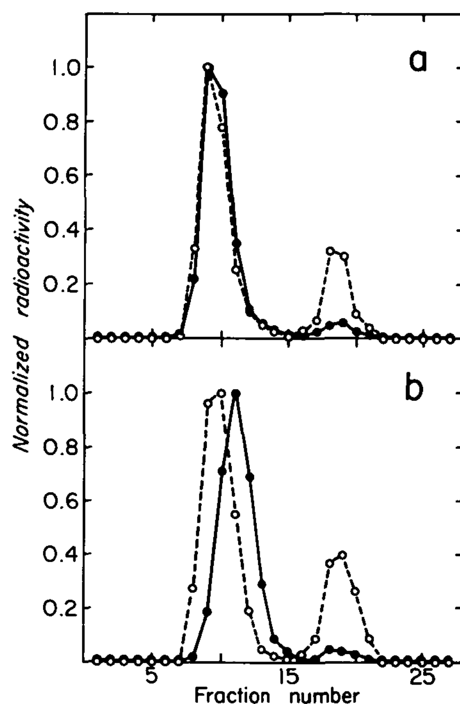

Figure 2: CsCl-propidium diiodide equilibrium analysis of the effects of DNA untwisting enzyme on SV40 nucleoprotein complexes. In (a), SV40 nucleoprotein complexes taken from Region III of Figure 1 were incubated with purified DNA untwisting enzyme. In (b), a reaction mix identical to (a) was incubated in the presence of 1 μg/ml of ethidium bromide. Reactions were stopped, deproteinized, dialyzed, and centrifuged as described in the text. In both reactions, the mole ratio of enzyme to DNA was 0.1 (●———● ^{3}H-labelled SV40 nucleoprotein complex DNA; o-----o ^{14}C-labelled SV40 DNA added just prior to centrifugation as a marker.)

density than native purified SV40 DNA in a CsCl-propidium diiodide gradient. When viral nucleoprotein complexes were incubated with untwisting enzyme in the presence of 1 μg/ml of ethidium bromide, the treated complex DNA banded at a lower buoyant density than did untreated marker SV40 DNA in a CsCl-propidium diiodide gradient (Figure 2b). This result suggests that the DNA in the complex is accessible to the untwisting enzyme. However, since the presence of ethidium bromide during the untwisting reaction could have altered the configuration of the chromatin, a second method was used to show accessibility of the DNA untwisting enzyme to the DNA in the chromatin.

Nicking of the chromatin DNA by the DNA untwisting enzyme. The DNA untwisting enzyme removes superhelical turns from circular DNAs through the

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introduction of a transient nick. One can measure the level of nicked intermediate by stopping the reaction with high pH (>12.5) and sedimenting the products through alkaline sucrose (16). In alkali, closed circular DNA denatures to a compact supercoiled form which sediments at 53 S, while nicked circular molecules denature to single-stranded circles and linears which sediment in a broad peak at 16-18 S (29, 30). Since the untwisting enzyme is inactive above 0.5 M NaCl (16), the addition of high salt to an untwisting reaction results in the disappearance of the nicked intermediate. This distinguishes nicking by the untwisting enzyme from the activity of any nucleases which may be present.

When viral nucleoprotein complexes from Region III were incubated with excess DNA untwisting enzyme at 23°C for 10 min followed by high pH treatment and alkaline sucrose sedimentation, the level of 16-18 S DNA increased from a background level of 19% to 48% of the total (Figure 3a). When the reaction was stopped by a high salt treatment (1 M NaCl) prior to alkaline denatur-

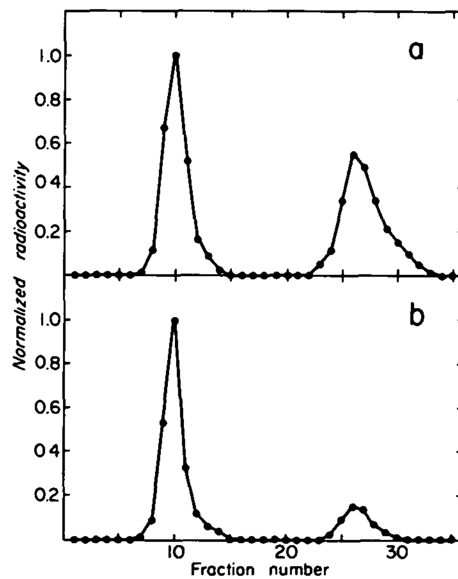

Figure 3: Alkaline sucrose sedimentation analysis of the effects of DNA untwisting enzyme on SV40 nucleoprotein complexes. In (a), SV40 nucleoprotein complexes taken from Region III of Figure 1 were incubated with purified DNA untwisting enzyme. The reaction was stopped with alkali, neutralized, treated with proteinase K and layered onto alkaline sucrose. In (b), the initial incubation was followed by treatment with 1 M NaCl for an additional 10 min prior to high pH exposure. Peak fractions contained (a) 281 cpm and (b) 436 cpm. In both reactions, the enzyme to DNA ratio was 4.5.

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ation, the level of 16-18S DNA returned to 19% (Figure 3b) which indicates that the observed nicking was due to the action of the untwisting enzyme and not the consequence of a contaminating nuclease. In other experiments (results not shown) the level of nicking of the chromatin DNA by the enzyme approached 92%. These results show that the DNA untwisting enzyme has access to sites on virtually every SV40 chromatin molecule.

DISCUSSION

In contrast to the observation of Sen and Levine (17) we failed to observe a significant association of the DNA untwisting enzyme with the purified SV40 nucleoprotein complex. This difference could be due to small variations in the two procedures employed for isolation of the viral chromatin. If one assumes that the untwisting enzyme from CV-1 cells does not differ significantly in molecular weight and specific activity from the enzyme purified from rat liver nuclei (23), one can calculate the maximum number of untwisting enzyme molecules per DNA molecule present in Region III of Figure 1. We feel this assumption is justified since crude extracts prepared from CV-1 nuclei yield a specific activity for untwisting enzyme within the range found for crude extracts prepared from rat liver nuclei (Young, unpublished). Based on molecular weights of 3.6×10^6 for SV40 DNA (31), and 66,000 for the DNA untwisting enzyme (23), the maximum ratio of untwisting enzyme molecules to SV40 DNA molecules in Region III in a representative experiment was 2×10^{-3}. This calculation indicates that, in our hands, the DNA untwisting enzyme is not preferentially associated with the SV40 nucleoprotein DNA. If untwisting enzyme had been associated with the complex with a stoichiometry of 1:1, we could have detected it with the DNA filter binding assay. We cannot rule out the possibility that the enzyme is bound in a form which is not active on the exogenous substrate DNA used in the usual *in vitro* assay.

If this observation is physiologically significant, then the failure to find untwisting enzyme in association with SV40 chromatin is somewhat surprising given the viral DNA's obvious need for a swivel during replication. If the enzyme does provide the swivel during replication, perhaps it is associated preferentially with replicative forms of SV40 DNA and dissociates quickly from completed viral chromatin. Preliminary evidence, obtained by analyzing six times as much nucleoprotein complex as used here, indicates that a small amount of untwisting activity does sediment slightly faster than the mature form of SV40 chromatin, near the region expected for the replicating molecules (Young, unpublished).

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In agreement with earlier studies, treatment of SV40 nucleoprotein complexes with the DNA untwisting enzyme does not result in a change in the superhelix density of the chromatin DNA. However, a 10% change in the number of superhelical turns (32) in the SV40 DNA would not have been detected by the CsCl-propidium diiodide analysis (33). When complexes were treated with untwisting enzyme in the presence of ethidium bromide, the complex exhibited a lower buoyant density than the marker DNA in a CsCl-propidium diiodide gradient indicating a change in superhelix density. Although this result suggests that sites are available for the untwisting enzyme to act, one cannot rule out the possibility that ethidium bromide is altering the complex and thereby creating sites for the untwisting enzyme to act. Benyajati and Worcel (34) report that ethidium binding to DNA in low salt does not displace histones; however, the effects of ethidium bromide intercalation on non-histone proteins and on overall chromatin structure have not been well characterized. We felt that an alternative approach which would keep the chromatin structurally intact and close to its native configuration was necessary in order to show that sites for the untwisting enzyme were present on the nucleoprotein. The presence of a significant level of nicked intermediate in the complexes treated with untwisting enzyme demonstrates that the nicking-sealing reaction of the untwisting enzyme is indeed occurring on the viral chromatin.

Taken together, these data imply that the regions of DNA within the nucleoprotein which are accessible to the DNA untwisting enzyme are under no topological strain. This observation indicates that the accessible DNA probably does not contain single-stranded loops due to topological constraints imposed by chromatin proteins. Crick (35) proposed that such loops could act as regulatory control sites for gene expression. If such regions exist within the chromatin DNA, our data indicate that they are inaccessible to the untwisting enzyme.

Theoretically, there are two possible sources for the negative superhelical turns found in purified SV40 DNA. The turns may be generated by the interaction of the DNA with histones to form the nucleosomes, and/or they may result from an activity analogous to the DNA gyrase recently identified in _E. coli_ by Gellert and his colleagues (36). If a DNA gyrase activity _in vivo_ predominates over a DNA untwisting enzyme, then the isolated SV40 chromatin should contain superhelical turns which can be relaxed by treatment with untwisting enzyme _in vitro_. Our observation that the superhelix density of the chromatin DNA is unaffected by the untwisting enzyme suggests that none

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of the observed superhelical turns are due to the action of a gyrase-like activity. Consistent with this conclusion is the finding that essentially all of the turns can be accounted for by the interaction of the DNA with the histones (18).

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