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Evidence for Variation of Supercoil Densities Among Simian Virus 40 Nucleoprotein Complexes and for Higher Supercoil Density in Replicating Complexes

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The distribution of DNA topoisomers in intracellular simian virus 40 DNA was analyzed by gel electrophoresis. The results suggested that DNA extracted from 70S chromatin had a different superhelical density distribution as compared with the DNA obtained from virions or virion assembly intermediates. The heterogeneity of simian virus 40 viral DNA superhelical density at a late time after infection was partly due to increased virion production and partly due to the intrinsic heterogeneity of the superhelical density of DNA extracted from virions. Using two-dimensional gel electrophoretic analysis we also showed that simian virus 40 DNA templates used for DNA replication have a higher average superhelical density than the bulk of intracellular viral DNA.

DNA supercoiling is a common feature of DNA in both procaryotic and eucaryotic cells. Both genetic and biochemical studies have suggested that supercoiling of DNA is important in the control of gene expression (for a review, see reference 14). Although the origin of supercoiling of procaryotic DNA is presently obscure, the study of simian virus 40 (SV40) minichromosomes by Germond et al. (5) has suggested that DNA supercoiling in eucaryotes arises by the winding of DNA around histone cores in nucleosomes. This simple concept of the origin of supercoils in SV40 DNA is, however, complicated by two observations. The first one is the so-called linking number problem which arises when the linking number change per nucleosome in SV40 minichromosomes is found to be far less than that expected from the coiling of DNA in nucleosomes studied by both physical and biochemical methods (for a review, see reference 17). The second problem associated with the supercoiling of SV40 DNA is the observation by several laboratories (5, 9, 13) that the distribution of SV40 DNA topoisomers analyzed by gel electrophoresis techniques is much more heterogeneous than could be accounted for by thermal fluctuation. Although the first problem has been the subject of several investigations and model building (7, 10, 15, 19), no explanation is yet available for the cause of heterogeneity of superhelical density in SV40 DNA. In the present study we sought to analyze the origin of the broad distribution of SV40 DNA topoisomers. Our results indicate that the heterogeneity of intracellular SV40 DNA supercoil density is partly due to the existence of several forms of intracellular SV40 nucleoprotein complexes (NPC) and originates in part from the intrinsic heterogeneity in virion DNA. We also show by two-dimensional analysis that SV40 DNA replication is initiated from a DNA population with higher superhelical density than the bulk of intracellular viral DNA.

MATERIALS AND METHODS

Materials. [^{3}H]thymidine (77 mCi/mmol) and En^{3}Hance were obtained from New England Nuclear Corp. Proteinase K and chloroquine diphosphate were purchased from Boehringer-Mannheim Biochemicals and Sigma Chemical Co., respectively.

Isolation of SV40 DNA and NPC. Infection of CV-1 cells with SV40 virus and pulse labeling of SV40 DNA have been described previously (1, 4). SV40 DNA was extracted from infected cells by a modification of the Hirt procedure (8). Infected cells were lysed on a tissue culture plate with the addition of lysis buffer containing 1% sodium dodecyl sulfate, 0.1 M NaCl, 10 mM Tris-hydrochloride, 1 mM EDTA, pH 7.4. The plate was rotated rapidly during and after the addition of lysis buffer to ensure rapid lysis. After 5 min at room temperature, NaCl was added to 1 M, and the viscous lysate was poured into 1.5-ml Eppendorf tubes. The lysates were frozen at −15°C for 30 to 40 min and spun for 15 min in an Eppendorf centrifuge in a cold room. The supernatant was digested with proteinase K at 200 μg/ml at 37°C for 1 h, and the DNA was extracted with phenol and chloroform as described previously (4).

SV40 NPC were extracted from infected cell nuclei as described previously (1, 4). Mature virus particles in the NPC extract were further purified by banding in a CsCl density gradient (4). For extraction of DNA from SV40 NPC fractions, solutions containing NPC were made 1% by rapidly mixing with the appropriate volume of 20% sodium dodecyl sulfate, digested with 200 μg of proteinase K per ml at 37°C for 1 h, and extracted with phenol and chloroform. For extracting DNA from mature virus two other methods were also used. Solution containing virus particles was mixed rapidly with sodium dodecyl sulfate and proteinase K at a final concentration of 2% and 200 μg/ml, respectively, incubated at 37°C for 1 h, and extracted with phenol and chloroform. Alternatively, virus solution was mixed rapidly with an equal volume of phenol and sodium dodecyl sulfate (final concentration, 2%). The aqueous phase was then reextracted with phenol and chloroform.

Gel electrophoresis. Electrophoresis analysis was performed in a flat-bed submerged gel system at 200 mA. To resolve DNA topoisomers in one-dimensional analysis, SV40 DNA samples were run in a 1 or 1.2% agarose gel in the presence of 50 to 75 μg of chloroquine per ml as described by Shure et al. (13) or in the presence of 0.016 μg of ethidium bromide per ml as described by Keller (9). Electrophoresis was also performed in a 1.8% polyacryl-

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amide–0.5% agarose mix gel without intercalating chemicals (9). Electrophoresis buffer was 40 mM Tris–5 mM sodium acetate–1 mM EDTA, pH 7.4 (TEA buffer).

For two-dimensional gel analysis of DNA topoisomers, DNA samples were run in the first dimension in a 1% agarose gel either without intercalating chemicals or in the presence of 5 to 12 μg of chloroquine per ml. The second-dimension analysis was performed in a 1.2% agarose gel in the presence of 75 to 100 μg of chloroquine per ml. Gels after electrophoresis were treated with En^{3}Hance and fluorographed according to the manufacturer's instructions.

RESULTS

Changes in superhelical density of intracellular SV40 DNA during the late cycle of infection. Analysis of the distribution of supercoil density in covalently closed circular DNA has been greatly facilitated by the development of the gel electrophoresis technique (9). This technique separates DNA topoisomers into a ladder of discrete bands, each different from its neighbor by one linking number (18). Shure et al. (13) improved this technique by using chloroquine as an unwinding agent. We used this technique to examine the distribution of DNA topoisomers in SV40 DNA synthesized at different times after the start of the late cycle of infection. With a concentration of 50 μg of chloroquine per ml, SV40 DNA was resolved into topoisomers with positive supercoil turns (13). The average negative superhelical density of SV40 DNA made during 18 to 22 h postinfection was higher than that of the 48- to 52-h postinfection sample, which in turn was more negatively supercoiled than the DNA synthesized between 68 and 72 h postinfection (Fig. 1). The difference in superhelical density between the samples of two time points and the samples of the two latter time points was approximately 4×10^{-3}. The results in Fig. 1 and 2 also show that the distribution of DNA topoisomers in the 18- to 22-h sample was rather homogeneous in distribution but became increasingly broader and asymmetric as infection proceeded from 18 to 72 h postinfection. There are 9 to 10 visible bands in Fig. 2, a distribution very similar to the limit product of nick-closing enzyme on free SV40 DNA in solution (13).

DNAs extracted from SV40 70S chromatin and virions differ in superhelical density. Since the observed decrease in superhelical density during infection correlated with the increased production of virus particles, a possible explanation is that the DNA in the virus particle has lower average negative superhelical density than the bulk of intracellular SV40 DNA. To examine this possibility, we extracted and separated different forms of intracellular SV40 NPC in sucrose gradients as described previously (4) and compared the distribution of DNA topoisomers present in different NPC fractions. DNA extracted from SV40 70S chromatin had lower electrophoretic mobility than the DNA extracted from the 150 to 210S virion and virion assembly intermediates in the presence of 50 μg of chloroquine per ml (Fig. 3). Since in this chloroquine concentration SV40 DNA is resolved into topoisomers with positive supercoil turns, the results shown in Fig. 3 indicate that SV40 DNA present in the 70S viral chromatin has higher average negative supercoil density than DNA in the 150 to 210S NPC, which contains both mature virus particles and virion assembly intermediates. Mature virus particles purified by CsCl density gradient banding gave a pattern almost identical to that of the 150 to 210S complexes. These results were confirmed by using electrophoresis conditions that resolved topoisomers with negative superhelical turns, such as electrophoresis in the presence of low concentration of ethidium bromide or electrophoresis in an agarose-polyacrylamide mix gel without intercalators as described above (data not shown). These results indicate that the change in supercoil density of SV40 DNA during infection as seen in Fig. 1 is due to the increased production of virion and virion assembly intermediates at later times in the infectious cycle.

SV40 DNA template for DNA replication has higher superhelical density than the bulk of DNA in 70S chromatin. The results described above show that DNA in 70S viral chomatin has higher average superhelical density than the DNA packaged or being packaged into capsid shells. Since 70S NPC itself is a mixture composed of NPC involved in DNA and RNA synthesis as well as the NPC that serves as the precursor of mature virus particles, each subpopulation of the 70S NPC could contain DNA with different superhelical densities. We were particularly interested in learning whether DNA templates used for DNA replication or transcription have superhelical densities different from those of the bulk of intracellular viral DNA. To analyze this problem we chose to study SV40 DNA involved in DNA replication for the following reasons. No methods have yet been developed for identifying the DNA template engaged in transcription in a two-dimensional gel system. On the other hand, DNA replication intermediates can be recognized by pulse labeling and by their characteristic mobility in two-dimensional gel elec-

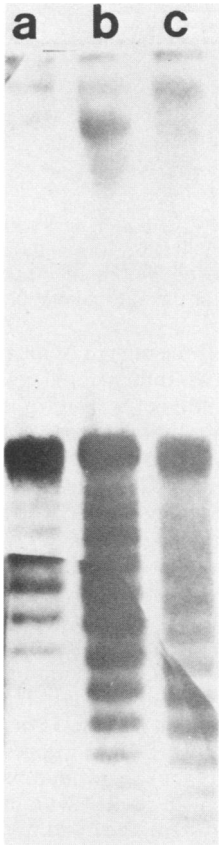

FIG. 1. Gel electrophoresis analysis of SV40 DNA isolated at different times after infection. SV40 DNA was labeled from 18 to 22 h (a), 44 to 48 h (b), and 68 to 72 h (c) postinfection with [^{3}H]thymidine, extracted by the Hirt method, and analyzed by gel electrophoresis in a 1.2% agarose gel in the presence of 50 μg of chloroquine per ml.

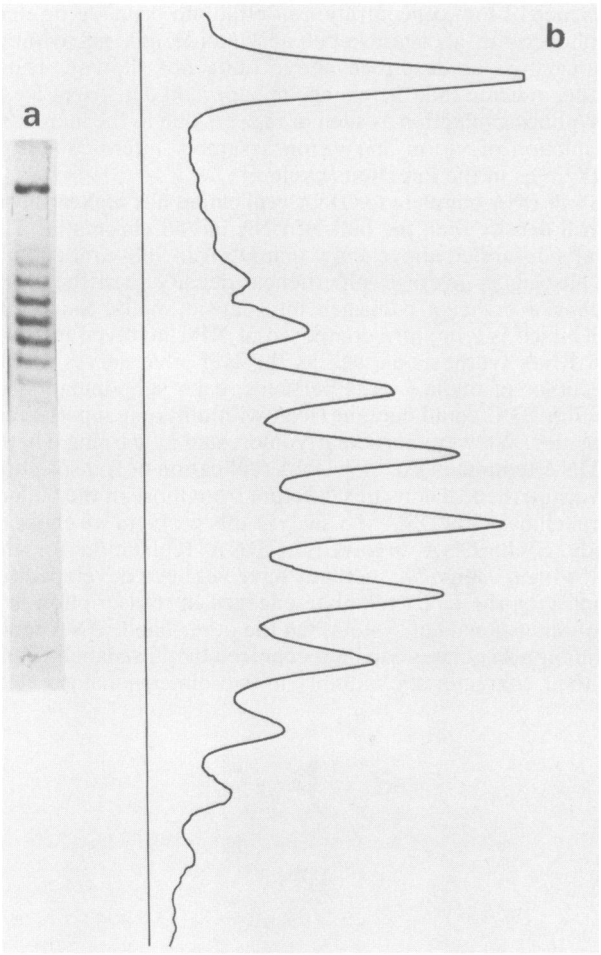

FIG. 2. Topoisomer distribution of SV40 DNA labeled from 18 to 20 h postinfection. SV40 DNA was analyzed in a 1.2% agarose gel in the presence of 75 μg of chloroquine per ml. (a) Autoradiograph. (b) Densitometer scan of the autoradiography shown in (a).

trophoresis. Two to three curves of nascent SV40 DNA have been observed in two-dimensional gel analysis when the replicating intermediate is resolved in the second dimension in alkaline denaturing buffer (6, 16). The curve (RI) that is bounded at one end by the position of supercoil form I DNA originates from the replicating intermediates containing covalently closed supercoiled parental strand DNA. The curve (RII) bounded on one side by nicked circular SV40 DNA is derived from replicating intermediates with a nick or nicks in the parental DNA. When the intact replicating intermediates were analyzed by a two-dimensional gel with both dimensions in neutral buffer but containing different amounts of chloroquine, two curves corresponding to RI and RII in the alkaline gel system were observed. The RI′ (corresponding to the RI curve in the alkaline gel) curve in the chloroquine two-dimensional gel (Fig. 4A) represents the replicating intermediates with intact supercoiled parental DNA strands, whereas the RII′ curve is RI′ with nicks in the parental DNA. In this gel system the mature DNA products, i.e., form I SV40 DNA, were resolved into a population of topoisomers. Assuming that SV40 DNA replication is a continuous process, we reasoned that the point or region of intersection between replication curve RI′ and the line or curve formed by mature SV40 DNA form I topoisomers in the second dimension should represent the SV40 DNA topoisomers from which SV40 DNA replication is initiated. When no chloroquine was present in the first dimension and the second dimension was performed in the presence of 75 μg of chloroquine to resolve DNA into positively supercoiled DNA species, replication curve RI′ could be seen to intersect with the line of form I DNA topoisomers at the region which is least positively supercoiled (Fig. 4B). Conversely, when the chloroquine concentration in the second dimension was reduced to 5 μg/ml to resolve SV40 form I DNA into topoisomers with negative supercoil, the replicating curve was found to intersect at the topoisomer species with the higher, if not the highest, negative supercoil density (Fig. 4A). If SV40 DNA were first resolved into both positively and negatively supercoiled species in the first dimension (10 μg of chloroquine per ml) and subsequently transformed into positively supercoiled species in the second dimension (75 μg of chloroquine per ml), replication curve RI could be extrapolated to intersect with the curve of form I DNA topoisomers at the region with the higher, if not the highest, negative superhelical density of pulse-labeled form I DNA (Fig. 4C). These results strongly suggest that SV40 DNA replication is initiated on SV40 DNA molecules with higher, if not the highest, supercoil density than the bulk of intracellular SV40 DNA. Similar conclusions were obtained when DNA extracted at 22 h postinfection was analyzed. Since pulse-labeled DNA extracted at this time of infection is almost exclusively present only in 70S viral chromatin, the DNA templates used for viral replication also had higher supercoil density than the average DNA in 70S chromatin. Thus, among the NPC in the 70S complexes, only those with higher topological tension were chosen for DNA replication.

DISCUSSION

The observation of heterogeneous distribution of SV40 DNA topoisomers has presented a paradox to the origin of supercoiling and organization of DNA in eucaryotic cells. The paradox, on the other hand, may provide an opportunity to analyze the factors that influence the DNA structure in cellular chromatin. In this study we sought the origin of SV40 superhelical density heterogeneity by analyzing the distribution of DNA topoisomers present in different intracellular forms of SV40 NPC. Based on the results in the present work we conclude that heterogeneity of superhelical density of intracellular SV40 DNA is partly due to differences in the DNA topoisomer distribution in various forms of intracellular NPC and is partly the result of the intrinsic heterogeneity of supercoil density in virion DNA. Our results confirm the previous observations in other laboratories that SV40 DNA superhelical density is heterogeneous (5, 9, 13). However, we differ from the previous work by demonstrating that when virion formation can be neglected, intracellular SV40 DNA is rather homogeneous and is higher in superhelical density than DNA in virus particles. Shure et al. (13) extracted intracellular DNA at 70 h postinfection, when the majority of the intracellular viral DNA is in either mature virus or assembly intermediates. It is therefore not surprising that they found little difference in the topoisomer distribution between intracellular and virion DNA.

A major conclusion of this study is that SV40 DNA linking number distribution in SV40 chromatin is rather homogeneous if virion formation can be neglected. The distribution of the DNA extracted at ca. 20 h postinfection (Fig. 2) is similar to the distribution of the limit product of nick-closing enzyme on free SV40 DNA in solution (13). This result suggests that most of SV40 chromatin is topologically relaxed and in equilibrium with the nick-closing enzyme in

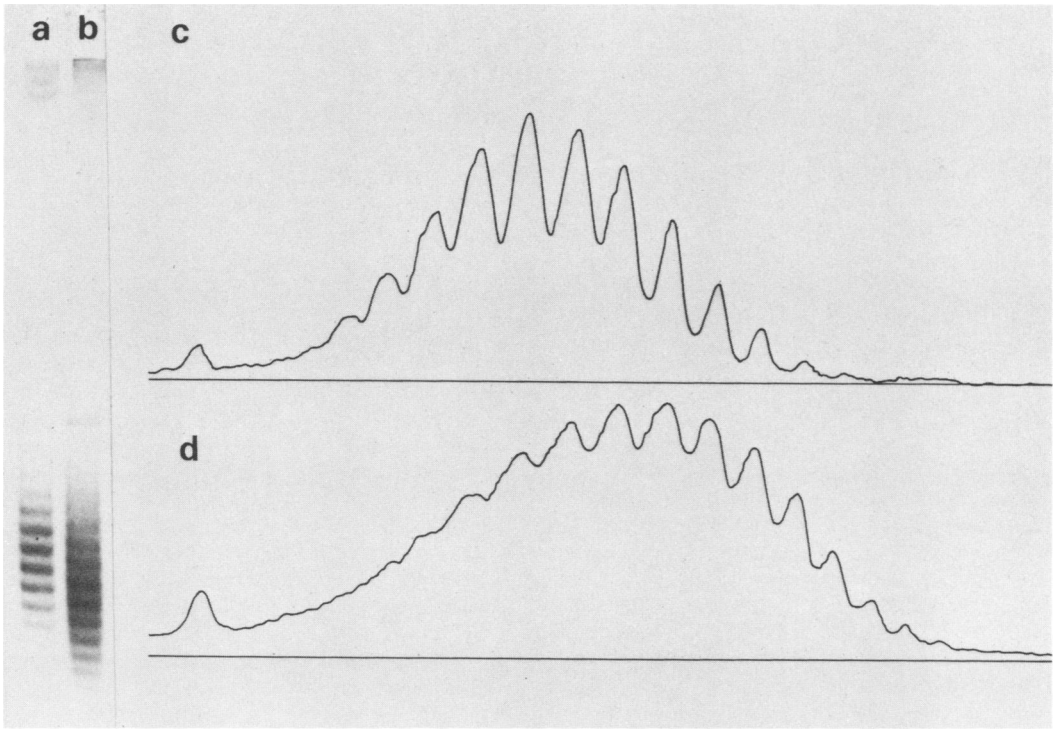

FIG. 3. Analysis of the distribution of DNA topoisomer extracted from (a) 70S chromatin and (b) 210S virion particles. Intracellular SV40 NPC were extracted as described previously (4) and fractionated by sucrose density centrifugation. Viral DNA present in the 70S chromatin peak and in the 210S virion particle peak in the sucrose gradient were extracted by phenol and chloroform and analyzed in a 1.2% agarose gel in the presence of 50 μg of chloroquine per ml. (c) and (d) Densitometric tracings of the gel autoradiographs shown in (a) and (b), respectively.

vivo. It also suggests that thermal fluctuation of SV40 chromatin must involve the whole molecule and not just the linker DNA alone, as suggested by Shure et al. (13). A model of SV40 chromatin with random orientation of nucleosomes was proposed by Grigoryev and Ioffee (7). Their calculation based on such a model showed that the standard deviation of the Gaussian distribution of linking number would be proportional to the square root of the number of nucleosomes. Such a model would seem to explain more closely the linking number and its distribution in SV40 DNA extracted from the 70S SV40 chromatin. Further analysis of the origin of linking number variation in SV40 chromatin could be obtained by studying the temperature dependence of the mean and the standard deviation of the Gaussian distribution of SV40 DNA extracted from infected cells at different temperatures. Such an approach was taken by Shure et al. (13), and they reported that no temperature dependence could be observed. Their failure to observe the temperature effect could be explained by the fact that most of the intracellular SV40 DNA they extracted was already in the virions, which obviously were inaccessible to the actions of topoisomerases. One would also have to take into consideration that equilibrium of linking number distribution in vivo may take a considerably longer period of time compared with DNA relaxation in vitro due to a limited number of topoisomerase molecules in the cells relative to SV40 chromatin. Taking advantage of our finding of the homogeneous distribution of SV40 chromatin at an early period of the late infection cycle, we are now investigating the temperature dependence of SV40 DNA topoisomerase distribution. Such an analysis would provide an estimation of the free energy coefficient of the in vivo process and therefore the nature of fluctuation of linking number in SV40 chromatin in vivo.

Although the distribution of SV40 DNA in 70S chromatin could be explained by the random conformational model of Grigoryev and Ioffee (7), the origin of heterogeneity of virion DNA superhelical density is still obscure at the present time. Since virions are derived from 70S viral chromatin, it is reasonable to assume that the heterogeneity is the result of the virion packaging process. Packaging of SV40 chromatin into virus particles necessarily involves extensive interaction between SV40 chromatin and capsid proteins. Our laboratory has already reported differences in nucleosome repeat length (3) and histone modifications (2) between SV40 chromatin and virion assembly intermediates. These biochemical alterations of packaged chromatin or even the direct interaction between capsid proteins and SV40 DNA could lead to the change of either the pitch of DNA double helix or the conformation of the packaged relative to the unpackaged SV40 chromatin. If a topoisomer I-like activity is present during the packaging process, those alterations in DNA structure would then become fixed and result in the shift of superhelical density of virion DNA relative to SV40 chromatin. The heterogeneity of topoisomer distribution could be explained by assuming a competition between topoisomerase activity and the virion packaging process. Those packaging intermediates that encounter less DNA topoisomerase during virion assembly would contain DNA with a superhelical density closer to that of the DNA in SV40 chromatin.

Our two-dimensional gel analysis suggested that SV40 DNA replication is initiated from the DNA template with a higher superhelical density than the bulk of intracellular DNA. This conclusion is based on the extrapolation of the replication curve in the two-dimensional gel toward 0% replication. Analysis using such an extrapolation approach

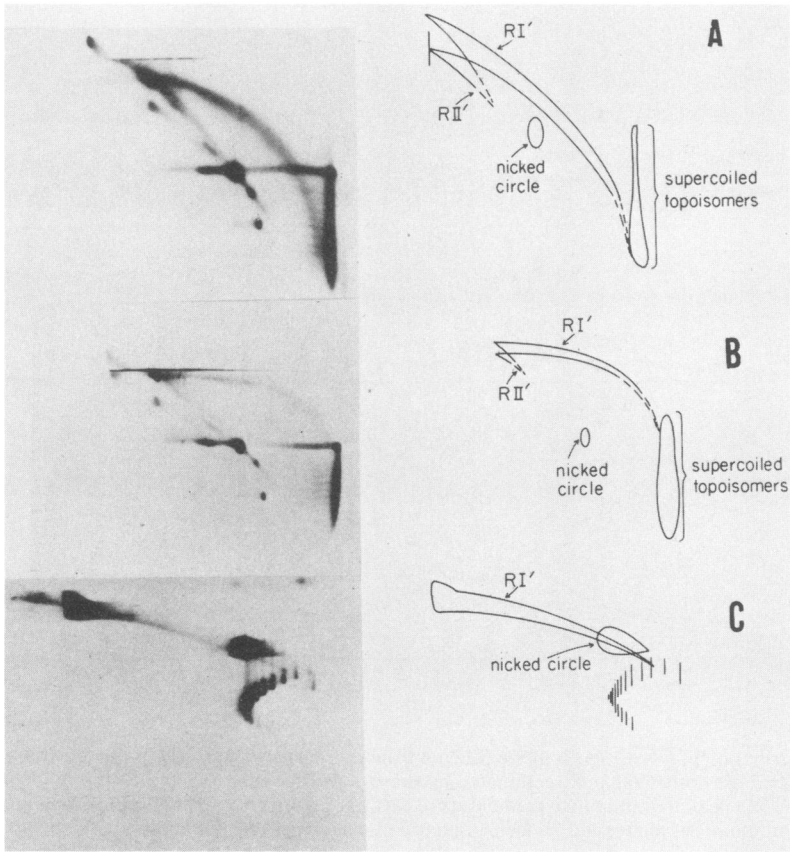

FIG. 4. Two-dimensional gel electrophoretic analysis of SV40 DNA replication intermediates. SV40 DNA pulse labeled for (A and B) 30 min and (C) 1 h were electrophoresed in the first dimension in 1% agarose (A and B) without chloroquine or (C) with 10 μg of chloroquine per ml and in the second dimension in a 1.2% agarose gel with 75 μg (B and C) or 5 μg (A) of chloroquine per ml.

necessarily assumes a continuous and smooth curve. This is supported by the data presented here and by previous work (6, 16). Our finding that SV40 DNA replication is initiated on DNA with higher superhelical density than the bulk of intracellular DNA suggests that topological strain may be important for SV40 DNA replication. Topological tension in SV40 DNA would facilitate the opening of the replication origin by the replication enzyme complex during the initiation step. A similar role of DNA supercoiling in transcription in vitro or in vivo has already been noted (11; see also a review in reference 14). The maintenance of a population of DNA with higher superhelical density presumably would require gyrase activity. Alternatively, the apparently higher superhelical density observed could be due to unwinding of SV40 DNA by an unwinding enzyme. In any case, the requirement of a DNA template with higher supercoil density for DNA replication could explain the results of Rinaldy et al. (12), who showed that superinfection of SV40-infected cells in the late cycle did not result in the immediate replication of the superinfecting viral DNA, even though all the replicating protein factors were presumably in full activity. As we show in this paper, virion DNA has the lowest supercoil density among all the intracellular viral DNA. The superinfecting virus therefore will contain too few templates with appropriate superhelical density for the initiation of SV40 DNA replication to be detected soon after infection.

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