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Superhelix Density Heterogeneity of Intracellular Simian Virus 40 Deoxyribonucleic Acid

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Covalently closed intracellular and viral simian virus 40 (SV40) deoxyribonucleic acid (DNA) were separately isolated from infected African green monkey cells (BSC-1) grown in culture. The two DNA species form overlapping bands centered at different positions in a propidium di-iodide-cesium chloride (PDI-CsCl) buoyant density gradient capable of separating closed DNA species with different superhelix densities. When the dense side of a ^{32}P-labeled intracellular DNA band was mixed with the light side of a ^{3}H-labeled intracellular DNA band and again centrifuged in a PDI-CsCl density gradient, two overlapping bands formed with modes displaced from each other. Similar band-splitting experiments performed with viral DNA always gave superimposable bands. The foregoing experiments demonstrate that the intracellular DNA is heterogeneous in superhelix density, whereas, by the same criteria, the viral DNA is homogeneous. The mean superhelix density of the intracellular closed DNA is approximately three-fourths as large as the superhelix density of the viral DNA. These results rule out the possibility that closed SV40 DNA is drawn randomly from the intracellular pool and assembled without a further nicking-closing step into virions. When the cells were grown and infected in the presence of ethidium bromide (EB), the intracellular closed DNA was found to be homogeneous in superhelix density and to have the same superhelix density as the viral DNA which, in turn, was unaffected by the presence of the drug. The foregoing results were explained by postulating that the intracellular DNA is formed with a homogeneous superhelix density and becomes heterogeneous in the absence of EB as a result of a nicking-closing cycle that occurs in a spacially or temporally heterogeneous environment. The drug EB would inhibit this action by inhibiting the nicking enzyme(s).

Buoyant density gradients containing intercalating dyes have proved to be effective for the detection and isolation of closed circular deoxyribonucleic acid (DNA; 4, 8). These density gradients have also been used in an analytical procedure for determining the superhelix density of closed DNA (2; H. B. Gray, Jr., and W. B. Upholt, *personal communication*). The superhelix density is defined as the number of tertiary turns per 10 base pairs (1). This quantity, when measured under standard conditions in which the duplex is in the Watson-Crick B form, is a direct index of the topological winding number α, a quantity which cannot be changed without nicking and again reclosing the molecule. The superhelix density σ_0, measured under standard conditions, varies from -0.01 to -0.11 among DNA species closed in vivo and in vitro with the polynucleotide ligase. The separation between the open and closed forms in a propidium di-iodide-CsCl (PDI-CsCl) gradient varies inversely with $|\sigma_0|$, decreasing from about 8 to 3 mm as the absolute value of the superhelix density changes from 0.01 to 0.08.

This communication presents an application of the buoyant separation method in an examination of closed simian virus 40 (SV40) DNA species formed in the course of the SV40 infection of African green monkey cells (BSC-1). These cells yield virus particles which contain only a portion of the total number of SV40 DNA molecules synthesized during the infectious cycle. The newly synthesized SV40 DNA accumulates as a pool within the cell prior to and during assembly of mature virions. Such a pool may represent a source of closed DNA for assembly into virions or, alternatively, the intracellular DNA may be nicked and reclosed before assembly occurs. It is

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also possible that the DNA molecules in the virions are never part of the intracellular pool. In this work we have found that superhelix densities of intracellular and viral closed DNA differ significantly. Moreover, the intracellular DNA contains molecules of various superhelix density, whereas the viral DNA appears to be homogeneous. These results alone rule out the possibility that closed DNA molecules are randomly selected from the pool of intracellular DNA for direct assembly into virions.

In another application of this method of superhelix density analysis (2, 4; H. B. Gray, Jr., and W. B. Upholt, *personal communication*; R. Espejo, E. Espejo-Canelo, and R. L. Sinsheimer, *personal communication*) have found that the superhelix density of closed PM2 DNA synthesized prior to formation of mature virions in *Pseudomonas* sp. BAL 31 cells infected with PM2 bacteriophage has a lower superhelix density than the viral DNA.

MATERIALS AND METHODS

The methods for cell growth, virus propagation and purification, and extraction of viral DNA were described previously (10).

Preparation of labeled DNA. Twenty-five μCi of ^{32}P-inorganic phosphate (International Chemical & Nuclear Corp., Irvine, Calif.) or 50 μCi of ^{3}H-thymidine (New England Nuclear Corp., Boston, Mass.) was added to single petri dishes containing BSC-1 cells infected with SV40 virus. The isotopes were added 12 hr after infection and at 24-hr intervals thereafter. Two and one-half to 3 days after infection, crude intracellular DNA was extracted with sodium deoxycholate by the procedures described by Bourgaux et al. (3) for polyoma-infected mouse embryo cells. The extract was stored at −20 C. At 7 days after infection, purified SV40 virus DNA was extracted as previously described (10). In certain experiments, the intracellular DNA was incubated with 100 μg of Pronase per ml at 60 C for 30 min and extracted with 1% sodium dodecyl sulfate (SDS) solution by the method used for extraction of DNA from mature virus. In addition, in some experiments, intracellular DNA was treated with 100 μg of ribonuclease per ml at 60 C for 30 min and then extracted with SDS.

Growth of infected cells in the presence of EB. At 1 hr after infection of BSC-1 cells with SV40 virus, 0.5 ml of ethidium bromide (EB) in Eagles medium (200 μg of EB per ml of Eagles medium) was added to a final concentration of 10 μg/ml, and the label was added as described above. At 24-hr intervals thereafter throughout the experiment, a further 0.5 ml of EB solution was added to the dishes.

PDI-CsCl gradients: preparative gradients. Samples of DNA from purified virus and intracellular extracts labeled either with ^{32}P or ^{3}H were added to a final volume of 3.0 ml of CsCl (density 1.51 to 1.52 g/ml)-PDI (500 μg of PDI per ml; Calbiochem, Los Angeles, Calif.) and centrifuged for 40 hr at 40,000 rev/min in a Spinco SW50.1 rotor at 20 C. Three-drop fractions were collected by direct tube puncture, and 10- to 50-μliter samples were applied to 2.5-cm discs (Whatman 3 MM). The discs were washed four times in ice cold 5% trichloroacetic acid, and dried in ethanol-ether prior to scintillation counting. Pure samples of closed intracellular and viral SV40 DNA were obtained as lower band material. The lower bands of labeled DNA were also divided into dense and light fractions by splitting the bands through the maxima.

PDI-CsCl gradients: analysis of superhelix density. Appropriately labeled mixtures of SV40 DNA samples were centrifuged in PDI-CsCl gradients as described above; 2-drop fractions were collected directly on Whatman 3 MM discs, acid-washed, and counted as before. Labeled, purified form II SV40 DNA was always added as a marker. PDI was used in these experiments instead of EB because the PDI-induced buoyant separations between closed and open DNA are about 1.8 times larger than those induced by EB (4).

RESULTS

Comparison of intracellular and viral SV40 DNA. Since SV40 virus particles are resistant to treatment with sodium deoxycholate, closed DNA isolated 60 to 70 hr after infection by means of extraction with deoxycholate represents some form of intracellular viral DNA. When this DNA was mixed with purified closed DNA from mature SV40 virus and a nicked SV40 marker DNA (Fig. 1) and centrifuged to equilibrium in a PDI-CsCl gradient, the intracellular DNA always formed a band at higher density than did the viral DNA. There are a number of possible reasons for such a difference in band position. Differential protein binding could cause this effect. However,

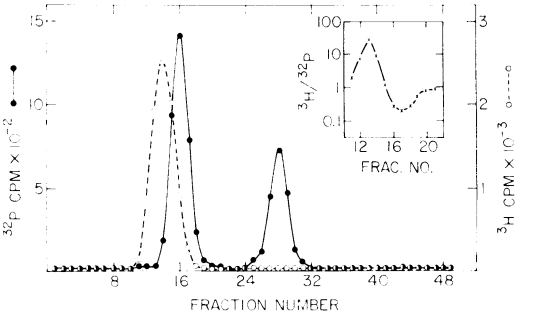

FIG. 1. *Buoyant behavior of a mixture containing closed and open ^{32}P-labeled viral SV40 DNA and closed ^{3}H-labeled intracellular SV40 DNA in a PDI-CsCl density gradient (1.52 g of solution per ml, 500 μg of PDI per ml) 20 C. The 3-ml sample was centrifuged for 40 hr in a SW50.1 rotor at 40,000 rev/min. The higher buoyant density of the intracellular DNA corresponds to a −20% change in the absolute value of the superhelix density. The insert displays the normalized ratio, $^{32}P/^{3}H$, across the closed bands.*

when the DNA fractions were treated with Pronase or extracted with SDS, the same differences in buoyant density were seen. Similarly, differential binding of ribonucleic acid (RNA) in the two types of DNA preparations was ruled out since ribonuclease-SDS treatment caused no change in the distributions observed. The DNA species might have different base compositions. The nicked forms, however, always cobanded, indicating no detectable difference in base composition.

The difference in band position is therefore attributed to a physical difference in the two sets of DNA molecules and, in this case, is considered to arise from differences in superhelix density between the DNA isolated from purified virus particles and from the intracellular SV40 DNA. The difference, $\Delta\sigma_0$, may be calculated with the relation

$$\Delta\sigma_0 = \sigma_0 - \sigma_0^* = 0.10[(r\Delta r/\bar{r}^*\Delta r^*) - 1] \quad (1)$$

obtained empirically by H. B. Gray, Jr., and W. B. Upholt (*personal communication*) for SV40 DNA in PDI-CsCl density gradients. In equation 1, Δr represents the distance separating the open and closed forms of a DNA, and $\bar{r}$ represents the mean of the distances from the center of rotation. The quantities with asterisks refer to a reference DNA banded in the same rotor and, in these experiments, in the same centrifuge tube. Since the distances between lower bands in our experiments were always less than 2 mm, equation 1 can be simplified with an error of less than 1%.

$$\Delta\sigma_0 = 0.10[(\Delta r/\Delta r^*) - 1] \quad (2)$$

The position of each band was taken to be the center of gravity of the appropriate radioactivity. The counts in each fraction were multiplied by the distance (expressed in units of fraction number) from an arbitrary reference external to the bands, summed, and normalized by the total number of counts in the band. In seven experiments the distance between the nicked DNA and the intracellular DNA was 9 ± 3% greater than the distance between the nicked DNA and the viral DNA. This effect indicates, according to equation 2, that $\Delta\sigma_{0,(I\text{-}V)}$ is $9 \pm 3 \times 10^{-3}$. Since the reference value of σ_0 for SV40 viral DNA is -0.039, the value of σ_0 for the intracellular DNA is -0.030. The absolute value of $\sigma_{0,I}$ is, therefore, smaller than the absolute value of $\sigma_{0,V}$. The physical displacements of the centers of gravity of lower bands relative to each other were approximately 0.7 mm. The superhelix density of the intracellular DNA, $|\sigma_0|_I$, is approximately three quarters as large as the superhelix density of viral DNA, $|\sigma_0|_V$.

Superhelix density heterogeneity in closed intracellular SV40 DNA. The band width at half-height of the intracellular DNA (Fig. 1) appears to be 20% larger than the corresponding width for viral DNA. This result suggests that there might be a greater degree of heterogeneity of superhelix density in the intracellular than in the viral DNA. Both of these DNA species were tested for superhelix density heterogeneity by means of experiments which we have called band-splitting experiments. Each DNA was separately labeled with ^{3}H-thymidine and with ^{32}P-inorganic phosphate. The four DNA species after isolation were separately centrifuged to equilibrium in PDI-CsCl density gradients. Aliquots of the fractions were counted, and each of the closed bands was split in two fractions through the band maximum. These fractions were designated as dense (D) and light (L) fractions. The appropriate isotope-labeled D and L fractions were then mixed and recentrifuged along with a nicked DNA marker in a PDI-CsCl density gradient. The mixture, $I_D + I_L$, of intracellular DNA (Fig. 2A) formed two nonsuperimposed bands of approximately equal band width. A similar result was obtained with a mixture of alternatively labeled I_D and I_L fractions. The mean displacement between the bands corresponded to a difference of $|\sigma_{0,D} - \bar{\sigma}_{0,L}|$ between the average superhelix densities of $7 \pm 2 \times 10^{-3}$. The fractional difference of approximately 25% is a rough indication of the extent of superhelix density heterogeneity in the intracellular DNA.

Superhelix density homogeneity of closed viral SV40 DNA. Band-splitting experiments were also performed with a set of four appropriately labeled D and L fractions obtained from viral DNA isolated from purified SV40 virus. The results obtained with one paired set of D + L fractions are presented in Fig. 2B. The bands superimpose closely, and the normalized ratio of isotopes across the band is constant. We conclude that viral SV40 DNA is homogeneous within the sensitivity of the band-splitting method.

Physical properties of closed SV40 DNA from BSC-1 cells grown in the presence of EB. EB added shortly after infection does not significantly reduce the yield of SV40 virus or of the total SV40 DNA (10). The apparent insensitivity to the drug is surprising in that the drug penetrates the nuclei in which the virus replicates. The nuclei, and especially the nucleoli, fluoresce strongly shortly after addition of the drug.

When intracellular closed DNA, I_{EB}, obtained from EB-treated cells was mixed with closed intracellular DNA, I, from untreated infected cells, and centrifuged to equilibrium in PDI-CsCl gradients, two overlapping but nonsuper-

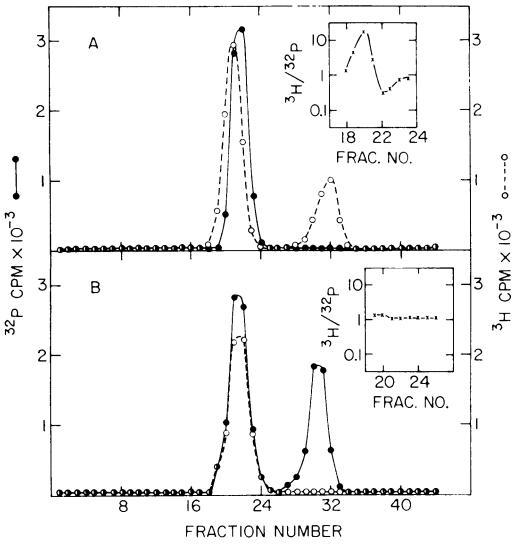

FIG. 2. *(A) A demonstration of superhelix density heterogeneity in intracellular SV40 DNA. The dense half of a ^{32}P-labeled intracellular DNA band, such as is displayed in Fig. 1, was mixed with the light half of a ^{3}H-labeled intracellular DNA band and centrifuged in a PDI-CsCl buoyant density gradient as described in Fig. 1. The separation between the closed bands here and in another comparable experiment corresponds to a difference between the two mean superhelix densities of 0.007 ± 0.002, compared with a value of −0.032 for the average superhelix density of intracellular DNA. (B) A demonstration of superhelix density homogeneity in viral SV40 DNA. A band splitting experiment as described in (A) was carried out with viral SV40 DNA. The normalized ratio of counts across the band, displayed in the insert, is substantially constant.*

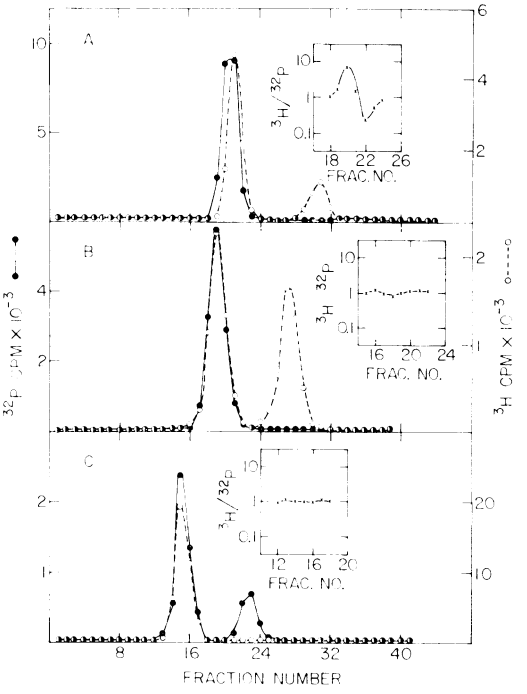

FIG. 3. *Effect of the presence of EB during the SV40 infection of BSC-1 cells on the buoyant behavior of the intracellular and viral SV40 DNA in PDI-CsCl density gradients. The experiments were carried out as described in the legend to Fig. 1. (A) ^{3}H-labeled intracellular SV40 DNA from EB-treated cells mixed with ^{32}P-labeled intracellular SV40 DNA from untreated cells. The separation between the two bands corresponds to an EB-induced increase in superhelix density of 0.007 ± 0.002. (B) ^{32}P-labeled viral SV40 DNA from EB-treated cells mixed with ^{3}H-labeled viral SV40 DNA from untreated cells. The constancy of the isotope ratio across the band as displayed in the insert indicates that the presence of EB has no effect on the superhelix density of viral SV40 DNA. (C) ^{3}H-labeled intracellular SV40 DNA from EB-treated cells mixed with ^{32}P-labeled viral SV40 DNA from untreated cells. The constancy of the isotope ratio across the band indicates that the effect of EB during infection is to cause the intracellular SV40 DNA to become homogeneous in superhelix density with a new value of σ_0 indistinguishable from that of viral SV40 DNA.*

imposed bands were always obtained (Fig. 3A). The corresponding mixture of viral DNA species (V + V_{EB}) gave completely superimposable bands (Fig. 3B). The mixture (I_{EB} + V) also gave completely superimposable bands (Fig. 3C). From these results we conclude that EB had no effect on the superhelix density of viral DNA but altered the population of intracellular DNA in two ways. The intracellular DNA becomes homogeneous in superhelix density and attains a value of σ_0 indistinguishable from that of the virus. The quantitative calculations for the superhelix density difference indicated in Fig. 3A, 7 ± 2 × 10^{-3}, is consistent with the previously indicated difference, 9 ± 3 × 10^{-3}, for intracellular and viral DNA species in the absence of EB. The homogeneity of both the viral and the intracellular DNA obtained from EB-treated cells was confirmed in band-splitting experiments presented in Fig. 4.

DISCUSSION

This work presents an application of a new and sensitive method for detecting differences in superhelix density among closed DNA species and, also, a method for detecting heterogeneity of superhelix density in a given closed DNA. The procedures described here involve buoyant banding of mixtures of differently labeled closed DNA species in CsCl density gradients containing the intercalating dye PDI, an analogue of EB.

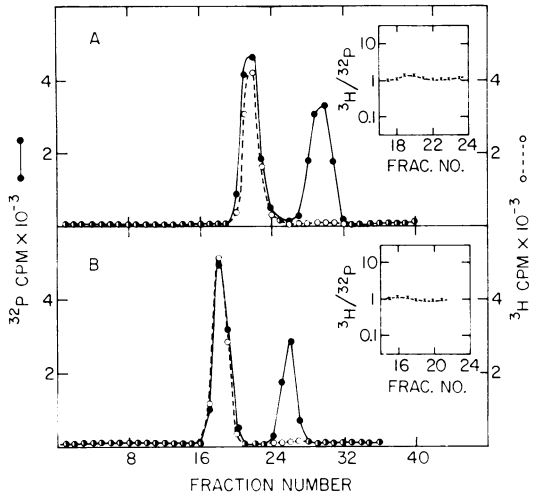

FIG. 4. *Demonstrations of superhelix density homogeneity in intracellular and viral SV40 DNA species formed in the presence of EB. (A) The dense half of a ^{32}P-labeled intracellular SV40 DNA was mixed with the light half of a ^{3}H-labeled intracellular SV40 DNA and centrifuged as described in the legend to Fig. 1. The constant ratio of isotopes across the band indicates that intracellular SV40 DNA formed in the presence of EB is homogeneous in superhelix density. (B) The dense half of ^{3}H-labeled viral SV40 DNA was mixed with the light half of a ^{32}P-labeled viral SV40 DNA and centrifuged as indicated above. The constant ratio of isotopes across the band indicates that viral SV40 DNA formed in the presence of EB is homogeneous in superhelix density.*

The method is based upon the qualitative observations of Hudson et al. (4) and quantitative, theoretical (2), and experimental (H. B. Gray, Jr., and W. B. Upholt, *personal communication*) studies of the dependence of the buoyant separation between open and closed DNA upon the superhelix density of the closed DNA. The latter studies employed photography of the fluorescent EB-DNA bands.

The superhelix density of a purified DNA in a standard solvent is a direct measure of an invariant property of a closed DNA which cannot be changed without opening and reclosing one of the backbone chains of the duplex. This property, the topological winding number α, is related to the number of tertiary turns τ and the number of duplex turns in the reference state β by the following equation:

$$\alpha = \tau + \beta \qquad (3)$$

The quantity α is most readily visualized as the number of times one strand of DNA winds about the other when the helix axis is constrained to lie in a plane. When the molecule is unconstrained, the DNA assumes its most stable structure with approximately 10 base pairs per turn, and superhelical turns are introduced in conformity with equation 3. The quantity τ is normalized by 10 times the number of base pairs to give the superhelix density, an intensive quantity.

$$\sigma \equiv \tau/\beta^{0} \qquad (4)$$

If $\beta = \beta^{0}$, the superhelix density is related to the invariant quantity α by the following relation:

$$\sigma = (\alpha/\beta^{0}) - 1 \qquad (5)$$

The quantity σ may be seen to measure directly the fractional deviation of the winding number of the duplex in the hypothetical "planar" molecule at the time of closure from the winding number of the same DNA having 10 base pairs per turn. At the time of closure, viral SV40 with $\sigma = -0.039$ was, therefore, shy 3.9% of the duplex turns that are present in the purified DNA in CsCl. Any change in σ, as was for example found in intracellular SV40 DNA, means that the intracellular DNA was closed for the last time with a different deficiency of turns: 3.0% instead of 3.9% for the viral DNA.

At the present time we are unable to provide a molecular explanation for the deficiency of turns that has been observed in all naturally occurring closed circular DNA species [W. Bauer and J. Vinograd. *In* F. E. Hahn (ed.), Progress in Molecular and Subcellular Biology, vol. 2, Springer-Verlag, New York, *in press*]. The possible explanations fall into two general nonexclusive categories. Nicked DNA is condensed into superhelical form and then closed. This type of process requires interaction with an organizing reagent, which may be part of the replication system, or the capsid protein in viral infection, or some other condensing agent. Alternatively, the DNA while open may be partially unwound by intercalators or other unwinding systems, which may include those mentioned above and, possibly, the transcription system also. It is against this background of uncertainty that the results obtained with SV40 DNA described earlier are discussed here.

We have found in the present work that intracellular DNA isolated 60 to 70 hr after infection is heterogeneous in superhelix density and the mean value of σ_0 differs from that of the DNA in virions isolated 170 hr after infection. The viral DNA appears to be homogeneous in superhelix density. When increasing amounts of EB are added to the infected cells (10 μg/ml, initially, and an additional 10 μg/ml added each day for 6 days), the superhelix density of the intracellular

DNA isolated at 2.5 to 3 days is homogeneous and indistinguishable from that of the virion DNA with a superhelix density unaffected by the presence of EB.

At the present time the infectious process of SV40 virus in monkey cells is under investigation in several laboratories. The published results (5, 6, 9) are still incomplete, and it is not possible to place our findings in the actual sequence of events. Instead, we outline (Fig. 5) a scheme for the infectious process which considers exclusively those steps involving the opening and closing of the circular duplex DNA.

We assign to the conceptual region, enclosed by a solid line in Fig. 5, all of the replicative processes that occur during the infectious process. It is assumed that these processes are semiconservative and, therefore, that the closed parental DNA is nicked to permit formation of daughter molecules. Replication of daughter molecules which have become part of the intracellular pool of closed DNA is also a possibility and is indicated by the dashed line from two distinguishable intracellular pools of closed DNA: one homogeneous, i_1, and the other heterogeneous, i_2. Several possible routes to virus are envisioned. The direct route aV bypasses intracellular pools and may be thought of as DNA synthesis occurring under the control of the encapsidation process. A second route, ai_1V, proceeds through a putative pool, i_1, homogeneous in σ. A third route, bi_2V, through the heterogeneous pool requires that a selection for the molecules with the right superhelix density occur or, alternatively, that the DNA be nicked and reclosed. Finally, more complex routes, ai_1i_2V and bi_2i_1V, which require an additional nicking-closing step need to be considered.

The heterogeneous pool i_2 is shown in this study to exist. To serve as a viral precursor, DNA from this pool must be nicked and reclosed so as to become homogeneous in σ, or a portion of i_2 having the right value of σ is selected to satisfy the requirements for viral assembly. The homogeneous pool of intracellular DNA i_1 is postulated without evidence as a possible but unnecessary intermediate in the viral-infected cell.

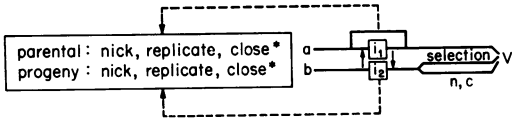

FIG. 5. *Diagrammatic illustration of several minimal pathways for the transfer of closed replicated DNA to mature virions.* (*)*The possibility that closure occurs after partial encapsidation has not yet been ruled out.*

When the infection occurs in the presence of EB, i_2 is absent and i_1 is present. Either paths bi_2 and ai_1i_2 are blocked, or path bi_2i_1 is stimulated so as to deplete i_2. The latter stimulatory action is difficult to conceive. On the other hand, the drug EB has been reported to inhibit pancreatic deoxyribonuclease activity (C. J. B. Tibbetts, *personal communication*) as well as polymerase activities in in vitro systems (7). The absence of the path bi_2 implies that heterogeneous DNA is not formed in the course of replication in the presence of EB. If this is the case, it is considered unlikely to be formed in the absence of EB. On the other hand, if upon replication only homogeneous DNA is made and a part of this material is released from the direct path to virus into i_1 and then into i_2, an inhibition of a single nicking-closing cycle in the step $i_1 \rightarrow i_2$ would account for the experimental result that the intracellular DNA becomes homogeneous in σ_0 and assumes the same value as the viral DNA. The foregoing reasoning suggests that, in the absence of EB, closed SV40 DNA of the correct superhelix density is made initially and that a part of this DNA is never part of the pool i_1. Another part, either synthesized prior to formation of capsid protein or synthesized in excess over the capsid protein, becomes pool i_1 which is then converted to i_2 as it accumulates in the cell.

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