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Integration of Simian Virus 40 Deoxyribonucleic Acid into the Deoxyribonucleic Acid of Primary Infected Chinese Hamster Cells

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Simian virus 40 deoxyribonucleic acid (DNA) became associated in an alkaline-stable form with the DNA of Chinese hamster embryo cells at 15 to 20 hr post-infection, at the time when cell DNA synthesis and T antigen were induced. The integration process was not inhibited by D-arabinosyl cytosine and was only partially inhibited by cycloheximide.

An association of simian virus 40 (SV40) deoxyribonucleic acid (DNA) with the cellular DNA of virus-transformed cells by alkali-stable covalent linkages has been demonstrated with some cell lines long after the transformation events have taken place (17). The possible relevance of the integration of a foreign genome to the oncogenic conversion of normal cells cannot begin to be understood without knowing the circumstances under which integration occurs with respect to the multiple changes which accompany, or are pathognomic of, transformation. In previous work, we have demonstrated that SV40 DNA is associated with the DNA of SV40-transformed Chinese hamster embryo (ChH) cells (8). We have also shown that in primary ChH cells, SV40 induces cellular DNA synthesis and that within one cell generation some cells undergo two periods of DNA synthesis with no intervening mitosis, which results in a tetraploid fraction of the cell population (13). This paper reports that integration of SV40 DNA into cellular DNA occurs in this cell system during the induction of cellular DNA synthesis, but that it is not dependent on it.

MATERIALS AND METHODS

Cell culture and virus. Primary and secondary cultures of ChH cells were prepared as previously described (13). Cl-21 is a cloned line of SV40-transformed ChH cells (8). SV40 (strain Rh 911) was grown in CV-1 cells (11). SV40 suspensions (4 ml in 60-mm petri dishes) were irradiated under a Westinghouse sterile lamp (782-L-20), delivering an energy of 17.8 ergs per mm^2 per sec at the irradiated surface.

Isolation of labeled and unlabeled DNA. ^{3}H-labeled SV40 DNA, unlabeled SV40 DNA, and cell DNA were isolated by a previously described procedure (8).

Isolation of nuclei. Nuclei were isolated by the Penman method (16) with the nonionic detergent Nonidet P-40 (NP-40).

Isolation of supernatant fluid DNA and sedimented DNA from nuclei of ChH cells infected with SV40. DNA from the nuclei of ChH cells infected with SV40 was isolated by a minor modification of the selective extraction method of Hirt (9). Briefly, isolated nuclei were lysed overnight in 5 ml of 0.6% sodium dodecyl sulfate (SDS), 0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.01 M ethylenediaminetetraacetate (EDTA), *p*H 8.0, at room temperature. After complete lysis, 5 M NaCl was added to a final concentration of 1 M. The sample was mixed slowly in a screw-cap centrifuge tube (capacity, 10 ml), stored at 4 C for at least 8 hr, and then centrifuged at 81,000 × *g* for 30 min in a Beckman Spinco 40 angle rotor at the same temperature. The sedimented pellet was dissolved in 5 ml of 1% SDS, 0.1 M Tris, 0.1 M NaCl (*p*H 9) and shaken with equal amounts of 80% distilled phenol at 4 C for 30 min. The resulting emulsion was separated into two layers by centrifugation at 11,000 × *g* for 10 min. The phenol treatment was repeated twice. The nucleic acids were precipitated by gently mixing the upper aqueous phase with two volumes of chilled ethanol and collected by centrifugation at 11,000 × *g* for 10 min. They were then dissolved in 3 ml of 1 × SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate), mixed with solid CsCl to a density of 1.70 g/cm^3, and centrifuged at 154,000 × *g* (Beckman Spinco SW 39 rotor) for 48 hr. The material recovered from the DNA band was designated "sedimented DNA" and used for DNA-ribonucleic acid (RNA) hybridization experiments.

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The supernatant fraction was precipitated with 2 volumes of chilled ethanol, dissolved in 5 ml of 1.0% SDS, 0.1 M Tris, 0.1 M NaCl (*p*H 9.0), and treated with phenol as described above. In some cases, the ethanol precipitate was treated with 5 ml of 0.3 N KOH for 2 hr at 37 C, neutralized to *p*H 7.0, and used as "supernatant fluid DNA" for DNA-RNA hybridization experiments.

Alkaline sucrose gradient. Nuclei were isolated from 2×10^7 to 3×10^7 cells by the Penman method (16) with yields varying between 15 and 25%. The nuclei were then suspended in 1 ml of RSB buffer (0.01 M Tris-hydrochloride, *p*H 7.4, 0.01 M NaCl, 0.0015 M $MgCl_2$) containing 1% NP-40 and 0.5% sodium deoxycholate and were layered on an alkaline sucrose gradient (10 to 30%) in 0.3 N NaOH, 0.01 M EDTA, 0.5 M NaCl (17). After storage at 4 C overnight to allow complete liberation of the DNA, the solution was centrifuged for 5 hr at 24,000 rev/min in a Beckman Spinco SW 25.1 rotor. The fractions were collected from the top by an ISCO density gradient collector, dialyzed, and subjected to DNA-RNA hybridization. The DNA was quantitated by the diphenylamine method of Burton (3). The DNA fractions were free from protein, as demonstrated in reconstruction experiments with ^{14}C-leucine- and ^{14}C-lysine-labeled cells.

SV40 complementary RNA. Tritiated SV40 complementary RNA (^{3}H-cRNA) was synthesized in vitro by *Escherichia coli* polymerase by a previously described method (8). Unless otherwise indicated, the same SV40 ^{3}H-cRNA was used throughout the experiments.

DNA-RNA hybridization. The technique used was similar to that described by Westphal and Dulbecco (19). Component I of SV40 DNA was converted into component II by treatment at 30 C for 60 min with 5×10^{-4} μg of deoxyribonuclease per ml in the presence of 0.01 M $MgCl_2$ and 30 μg of crystallized bovine serum albumin. The deoxyribonuclease-treated DNA was poured into 2 volumes of boiling water, boiled for 15 min, and rapidly chilled in ice; SSC was added to a final concentration of 6×. The cellular DNA was denatured by treatment with alkali (*p*H 12.8) for 10 min and then neutralized with HCl.

The denatured cellular DNA (10 to 15 μg/ml) was adjusted to 6 × SSC (final concentration) and slowly passed through a nitrocellulose membrane filter (BAC-T-FLEX, B-6; Schleicher and Schuell, Keene, N.H.) which had been soaked in 6 × SSC. The amount of cellular DNA initially adsorbed to the filter was calculated by measuring the optical density of a denatured DNA solution at 260 nm before and after passage through a membrane filter. Ninety to 100% of the denatured SV40 ^{3}H-DNA was adsorbed to the membrane. The membrane filter was dried, first at room temperature under vacuum overnight and then at 80 C for 4 hr, and then incubated for 20 hr at 65 C in a vial containing, in 1 ml of 6 × SSC, tritiated cRNA (10^5 to 2×10^5 counts/min) and 1 mg of SDS. After the hybridization, the vial was kept at room temperature for 3 hr; the filter was then washed in 2 × SSC and incubated for 1 hr at 35 C in 1 ml of 2 × SSC containing 10 μg of pancreatic ribonuclease. The filter membrane was washed by the passage of 2 × SSC through the membrane and then dried. Radioactivity was counted in a toluene-based scintillation mixture by a Beckman liquid scintillation spectrometer. During the hybridization, up to 10% of the cellular DNA (10 to 150 μg) immobilized on the membrane was released into solution. There was no evidence of release of SV40 ^{3}H-DNA (0.001 to 0.1 μg per membrane) from the filter, even after incubation for 48 hr at 65 C.

Assay of DNA synthesis and T antigen. Incorporation of ^{3}H-thymidine was determined, and immunofluorescence assay for SV40 T antigen was done as previously described (13). ^{3}H-thymidine at 0.2 μCi/ml was used for continuous labeling experiments, and 2 Ci/ml was used for pulse experiments.

Assay of ^{3}H-labeled amino acid incorporation in protein fraction. After cell monolayers were infected on small petri dishes at a multiplicity of infection (MOI) of 30, ^{3}H-labeled lysine, leucine, and valine were added to the medium (0.7 μCi/ml). At various times thereafter, the cells were collected by EDTA-trypsin treatment after repeated washing with phosphate-buffered saline. The cell pellets were then treated with 5% trichloroacetic acid, stored in ice for 1 hr, and boiled for 10 min. The precipitate was collected on the filter and washed with 5% cold trichloroacetic acid, and the radioactivity was counted as above.

Materials. Cytidine-5′-triphosphate-*5-^{3}H* (13.5 Ci/mmole), thymidine-*methyl-^{3}H* (6.0 Ci/mmole), L-valine-*2,3-^{3}H* (18.5 Ci/mole), L-leucine-*4,5-^{3}H* (58.1 Ci/mmole), and L-lysine-*4,5-^{3}H* (28.0 Ci/mmole) were all obtained from Schwarz BioResearch, Orangeburg, N.Y. D-Arabinosyl cytosine (Ara-C) was from the Upjohn Co., Kalamazoo, Mich., and cycloheximide (Acti-Dione) was from Calbiochem, Los Angeles, Calif.

RESULTS

Association of SV40 DNA with nuclear DNA. Nuclei from monolayers of primary ChH cells infected at an MOI of 30 were isolated at various times after infection and extracted for DNA by the Hirt method (9). DNA was isolated from the supernatant fluid and sedimented fractions and subjected to hybridization with SV40 ^{3}H-cRNA.

The association of the viral DNA with nuclear DNA began to be detectable at the time (10 to 15 hr postinfection) when cellular DNA synthesis occurred (Fig. 1). The proportion of the DNA hybridizable with SV40 ^{3}H-cRNA increased rapidly, but a plateau was reached at about 30 hr postinfection. The amount of supernatant nuclear DNA hybridizable with SV40 ^{3}H-cRNA decreased very rapidly, so that by 30 hr no free SV40 DNA could be detected by this method. However, when DNA was extracted from whole ChH cells infected by SV40, rather than from the isolated nuclei, a large amount of supernatant fluid DNA hybridizable with SV40 ^{3}H-cRNA

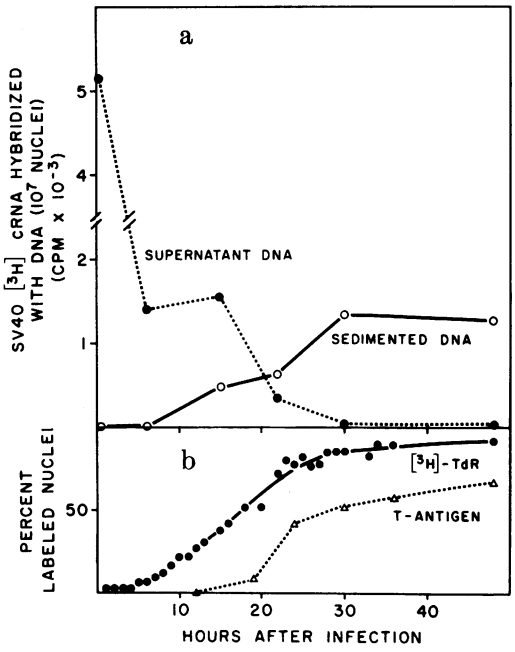

FIG. 1. *Chronological sequence of distribution of supernatant fluid and sedimented nuclear DNA hybridizable with SV40 ^{3}H-cRNA (a) and induction of cellular DNA and T antigen (b). (a) Confluent monolayers of primary ChH cells in two 1-liter Blake bottles were infected with SV40 (MOI of 30) and split into four 1-liter Blake bottles. At various times thereafter, nuclei were isolated and subjected to the selective extraction method of Hirt. The sedimented DNA (○) and supernatant fluid DNA (●) in the nuclei were extracted and hybridized with SV40 ^{3}H-cRNA (1.27 × 10^5 counts/min) in 1 ml of 6 × SSC containing 0.1% SDS. The counts per minute of the SV40 ^{3}H-cRNA hybridized with sedimented DNA were normalized to an average of 100 μg of DNA, which corresponds to the DNA content of 10^7 nuclei. The actual amount of DNA immobilized on the filter varied between 90 and 120 μg. From the observed counts per minute, the following counts per minute were subtracted: (1) the amount of SV40 ^{3}H-cRNA bound to uninfected ChH DNA (377 counts per minute per 100 μg of DNA) and (2) 20% of the SV40 ^{3}H-cRNA hybridized with supernatant fluid DNA, since this is the maximum amount of supernatant fluid DNA which may contaminate the sedimented DNA as calculated in reconstruction experiments with the addition of a known amount of SV40 ^{3}H-DNA. (b) Assays of DNA synthesis (●) and T antigen (△).*

could be detected at any time after infection (Fig. 2). To avoid residual cytoplasmic SV40 DNA in all of the following experiments, isolated nuclei were used.

The fact that association of the SV40 DNA with the sedimented fraction of nuclear DNA occurs at the time cellular DNA synthesis begins

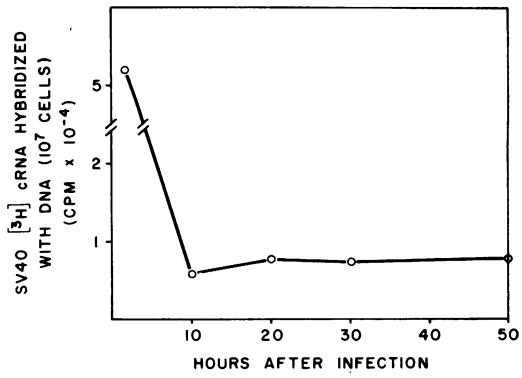

FIG. 2. *Amount of supernatant fluid DNA hybridizable with SV40 ^{3}H-cRNA from whole ChH cells infected with SV40. At various times postinfection, the supernatant fluid DNA was extracted from 10^7 ChH cells infected with SV40 and subjected to DNA-RNA hybridization as described in Fig. 1a.*

in the infected cells could indicate a causal or just chronological relationship. To resolve this question, the same experiments, that is, with the same number of cells infected at the same MOI, were performed in the presence of an inhibitor of DNA synthesis. Ara-C (15 μg/ml) was added to the cultures immediately after virus adsorption and maintained until harvest of the cells. Under these conditions, DNA synthesis during the period from 0 to 48 hr was inhibited by 95% in the drug-treated cultures as compared to the controls, whereas there was practically no inhibition of T-antigen induction (Table 1), as determined by the percentage of immunofluorescent cells. As shown in Fig. 3, the proportion of DNA hybridizable with SV40 ^{3}H-cRNA in the sedimented nuclear DNA was no less than in the untreated cultures (Fig. 1).

The effect of cycloheximide on the association of SV40 DNA with cellular DNA was then examined by the same method. Under these conditions, within 24 hr postinfection cycloheximide (5 μg/ml) inhibited, by more than 97%, the incorporation of ^{3}H-amino acid into trichloroacetic acid-precipitable material as well as T-antigen formation (Table 1). However, the association of SV40 DNA with sedimented cell DNA was only partially inhibited.

The amount of SV40 ^{3}H-cRNA hybridizable with the sedimented nuclear DNA was related to the MOI (Table 2); its decrease was proportional to the decrease of cells positive for T antigen. No association of SV40 DNA with nuclear DNA was detected when the MOI was 0.3, a multiplicity at which 2% of the cells were positive for T antigen.

Since functions of the viral genome can be

TABLE 1. *Effects of D-arabinosyl cytosine and cycloheximide on T-antigen formation, ^{3}H-thymidine incorporation, and the incorporation of labeled lysine, leucine, and valine*

Determination	Cells positive for T antigen (%)[a]		^{3}H- thymidine incorporation (counts/min)[b]		^{3}H-lysine, -leucine, -valine incorporation (counts/min)[c] at 24 hr
	24 hr	48 hr	24 hr	48 hr	
Control	81.5	95–100	904 (100)[d]	7,614 (100)	28,841 (100)
D-Arabinosyl cytosine (15 μg/ml)	66.5	95–100	24 (2.6)	432 (5.7)	
Cycloheximide (5 μg/ml)	0	0			840 (2.9)

[a] Percentage of cells positive for T antigen was determined by examining 250 to 500 cells.

[b] After adsorption of SV40, 2 μCi of ^{3}H-thymidine and D-arabinosyl cytosine was added to 10 ml of medium in a 60-mm petri dish containing two cover slips. At various intervals, the radioactive medium was removed and the cells were washed twice and incubated in fresh medium, with or without D-arabinosyl cytosine, for 1 hr. The cover slips were washed in PBS and fixed in Carnoy's solution. The values were based on the averages of counts from four cover slips.

[c] Values were determined for the cells in 60-mm petri dishes.

[d] Values in parentheses were expressed as percentages.

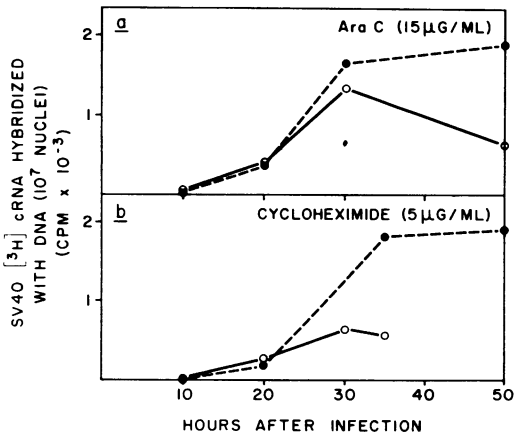

FIG. 3. *Effect of Ara-C and cycloheximide on the association of SV40 DNA to the sedimented nuclear DNA. After 1.5 hr of adsorption of SV40 (MOI of 30), the confluent primary cultures in two 1-liter Blake bottles were split into four 1-liter Blake bottles, and (a) D-arabinosyl cytosine (15 μg/ml) or (b) cycloheximide (5 μg/ml) was added to the cultures. Other details are the same as given in the legend for Fig. 1a. Symbols: (a) ●, no inhibitor; ○, Ara-C; (b) ●, no inhibitor; ○, cycloheximide.*

differentially inactivated by ultraviolet (UV) irradiation (5), the effect of UV irradiation on the capacity of the SV40 DNA to become associated to the "sedimented" cell DNA was determined. As an index of the intracellular penetration of the virus in these experiments, the amount of SV40 ^{3}H-cRNA hybridizable with the supernatant fluid DNA extracted from the isolated nuclei at 6 hr postinfection was determined. Association of SV40 DNA with cellular DNA, as well as the proportion of cells positive for T antigen

TABLE 2. *Effect of multiplicity of infection on the association of SV40 DNA with nuclear DNA*

Multiplicity of infection (PFU/cell)[a]	T-antigen-producing cells (%)[b]	Association of SV40 DNA with nuclear DNA (counts/min)[c]
30	100	3,548
3	15	470
0.3	2	0

[a] PFU, plaque-forming units.

[b] At 48 hr postinfection, based on counts of 250 to 500 cells.

[c] A 100-μg amount of nuclear sedimented DNA isolated from SV40-infected primary ChH cells at 48 hr postinfection was hybridized with SV40 ^{3}H-cRNA (1.6×10^5 counts/min). The value for normal ChH cell DNA (311 counts/min) was subtracted from the observed values.

and of cell stimulation into DNA synthesis, decreased exponentially at similar rates as a function of the length of UV irradiation (Fig. 4). The capacity of the virus to penetrate into the nuclei, however, was not impaired even after 60 min of irradiation. At 48 hr postinfection with a sample irradiated for 60 min, no SV40 DNA, free or associated, could be detected in the nuclei, indicating that the SV40 DNA from UV-inactivated virus was degraded between 6 and 48 hr postinfection.

Evidence that the viral DNA is integrated into cell DNA with alkali-stable covalent linkage. For a critical interpretation of the experiments to demonstrate alkali-stable covalent linkages of SV40 DNA to cellular DNA under conditions in which free SV40 DNA is present, it is necessary to determine whether free SV40 DNA may contaminate the high-molecular-weight cell DNA.

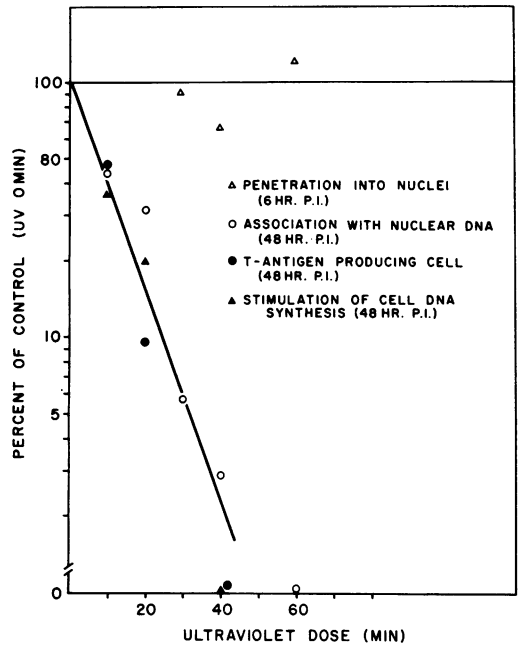

FIG. 4. *Effect of UV irradiation on the association of SV40 DNA with ChH nuclear DNA. Confluent monolayers of ChH cells were infected (MOI of 40) with SV40, which had been UV-irradiated for the indicated time. The rate of loss of infectivity was 2.5 $\log_{10}$ plaque-forming units per 20 min. The penetration of SV40 (Δ) was assayed by hydridization of SV40 ^{3}H-cRNA with the supernatant fluid DNA isolated from the nuclei at 6 hr postinfection. The value obtained in the controls with nonirradiated virus was 2,085 counts/min (100%). The association of SV40 DNA with sedimented nuclear DNA at 48 hr postinfection* (○) *was assayed. The value of SV40 ^{3}H-cRNA bound to the sedimented cellular DNA after infection with nonirradiated virus was 1,165 counts per min per 100 μg of cellular DNA (100%), after subtraction of the amount bound to normal ChH DNA (373 counts per min per 100 μg). At 48 hr postinfection with nonirradiated virus, the percentage of cells positive for T antigen* (○) *was 87%; the cumulative percentage of cells that had incorporated ^{3}H-thymidine (0.1 μCi/ml for 48 hr) was 70%, whereas the percentage in uninfected cultures was 35%. The values observed after irradiated virus infection were normalized to those from the unirradiated virus-infected cultures.*

For this purpose, the following preliminary experiments were done.

(i) Different amounts of nuclei (10^6, 5×10^6, and 10×10^6) were mixed with a constant amount of SV40 ^{3}H-DNA (2×10^4 counts/min; specific activity, 1.55×10^6 counts per min per μg, that is, 0.0144 μg) and then layered on an alkaline sucrose gradient. The SV40 ^{3}H-DNA appeared to be efficiently separated from the cellular DNA in all three samples. Figure 5a shows the distribution in the gradient when 5×10^6 nuclei were used. In these experiments, the total amount of SV40 DNA contaminating the cell DNA fractions (the last 10 tubes in the gradient) was found to be of the order of 0.000378 μg (total counts, 597 counts/min, that is, 3% of the input DNA).

(ii) Isolated nuclei (5×10^6) were mixed with two different amounts of SV40 DNA (0.06 and 0.6 μg, respectively), and, after centrifugation, fractions from each gradient were pooled in six samples and each was hybridized with SV40 ^{3}H-cRNA. In both gradients, a very small amount of DNA hybridizable with SV40 ^{3}H-cRNA was found in the high-molecular-weight fractions (last three samples of the pooled fractions, >100*S*; Fig. 5b), that is, 2.9% of the total.

(iii) Nuclei (6×10^6) isolated at 4 hr postinfection (MOI of 30) were layered on alkaline sucrose gradient, and the distribution of DNA which hybridized with SV40 ^{3}H-cRNA was determined. In this experiment (Fig. 5c), no DNA hybridizable with SV40 ^{3}H-cRNA was demonstrated in the high-molecular-weight DNA fractions. Thus, in both types of reconstruction experiments, when the input of SV40 DNA varied between 0.0144 and 0.6 μg, about 3% was found to contaminate the fast-sedimenting DNA. It should be noted that no contamination was found when the nuclei were isolated at 4 hr postinfection, and the results of the following experiments can be interpreted on this background.

Secondary cultures of ChH cells were infected at an MOI of 30 and CV-1 cells at an MOI of 10; between 3×10^6 and 7.5×10^6 cells were used for each gradient. As shown in Fig. 6b, at 36 hr after infection with SV40, the nuclei of the permissive CV-1 cells contained a large amount of low-molecular-weight DNA hybridizable with SV40 ^{3}H-cRNA, which corresponded to the position in the gradient of the SV40 DNA, with a very small hybridizable high-molecular-weight DNA fraction. With ChH cells at 8 hr postinfection, most of the DNA hybridizable with SV40 ^{3}H-cRNA (75%) was still present in a low-molecular-weight form (Fig. 6c); however, at 30 hr postinfection, the hybridizable DNA (90%) was almost totally associated with high-molecular-weight cellular DNA by alkali-stable linkage (Fig. 6d). It was also found, in cultures treated with Ara-C or cycloheximide, that most of the DNA hybridizable with SV40 ^{3}H-cRNA was linked to high-molecular-weight DNA (Fig. 6e and f). The fact that some low-molecular-weight DNA was hybridizable with SV40 ^{3}H-cRNA could result from fragmentation of cellular DNA or free SV40 DNA.

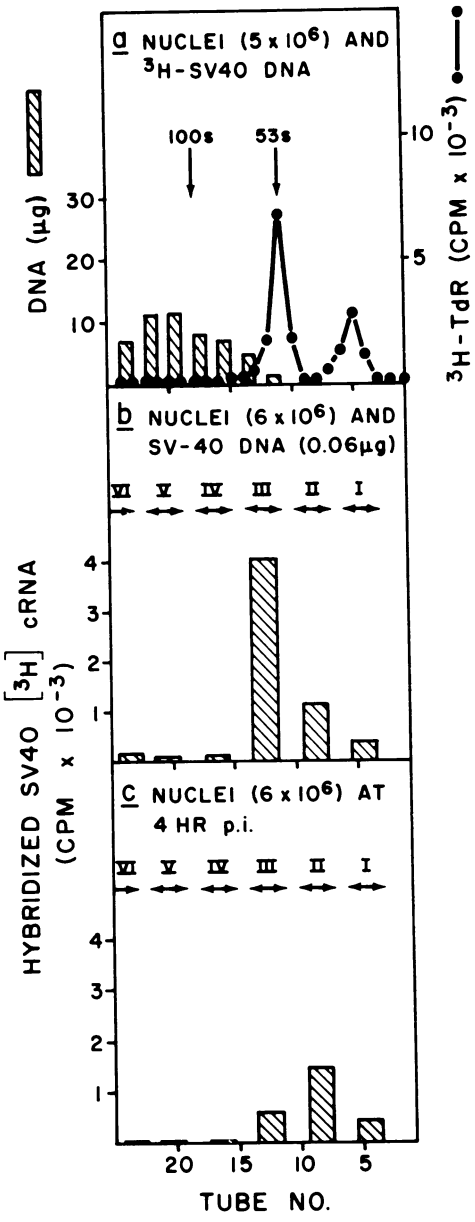

FIG. 5. *Velocity sedimentation of SV40 DNA and nuclear DNA in alkaline sucrose gradient. Different mixtures of SV40 DNA and nuclei were layered on an alkaline sucrose gradient (10 to 30%) and centrifuged for 8 hr at 83,000 × g. The samples of nuclei and DNA layered on the gradient were: (a) a mixture of SV40 ³H-DNA (2 × 10⁴ counts/min; 1.55 × 10⁶ counts per min per mg) and 5 × 10⁶ nuclei isolated from primary ChH cells; (b) a mixture of SV40 DNA (0.06 mg) and 6 × 10⁶ ChH nuclei. Fractions from each portion were pooled (as indicated in the graph by roman numerals) and hybridized with SV40 ³H-cRNA. (c) Nuclei (6 × 10⁶) isolated from SV40-infected (MOI of 30) ChH cells at 4 hr postinfection. Each sample of pooled fractions as described in (b) was hybridized with SV40 ³H-cRNA.*

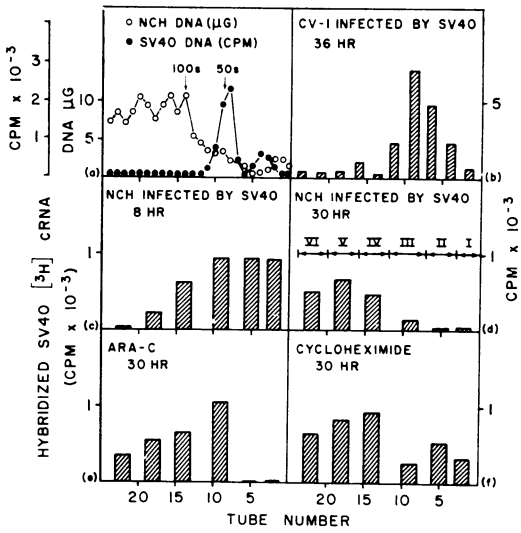

FIG. 6. *Size distribution of cellular DNA and SV40 DNA hybridized with SV40 ³H-cRNA in alkaline sucrose gradient. Nuclei were isolated from various cells and layered on alkaline sucrose gradient (10 to 30%) and then centrifuged for 5 hr at 83,000 × g. The samples of nuclei or DNA layered on alkaline sucrose gradient were (a) nuclei from normal primary ChH cells and a mixture of SV40 ³H-DNA component* I *(6 × 10³ counts/min) and component* II *(2 × 10³ counts/min); (b) nuclei from CV-1 cells at 36 hr postinfection (MOI of 10); (c) nuclei from ChH cells at 8 hr postinfection (MOI of 30); (d) nuclei from ChH cells at 30 hr postinfection (MOI of 30) in the presence of (e) Ara-C (15 μg/ml) and (f) cycloheximide (5 μg/ml). Fractions from each portion were pooled as indicated and hybridized with SV40 ³H-cRNA.*

Extent of the homology between SV40 ³H-cRNA and sedimented DNA in nuclei isolated from infected cells. The previous experiments indicated that some homology exists between SV40 ³H-cRNA and sedimented DNA extracted from the nuclei of infected cells. However, they do not provide any information about the extent of the complementary nucleotide sequence. Therefore, we investigated the heat stability of the DNA-RNA complexes. The DNA-RNA complexes on the nitrocellulose membrane filter were treated with ribonuclease and washed in 2× SSC. The filters were then treated with 1 ml of 2× SSC for 10 min at the indicated temperature. The eluate from the filter was counted (Fig. 7). The heat stability of DNA-RNA hybrids was essentially the same for (SV40-DNA plus SV40-cRNA), (Cl-21-DNA plus SV40-cRNA), and (sedimented

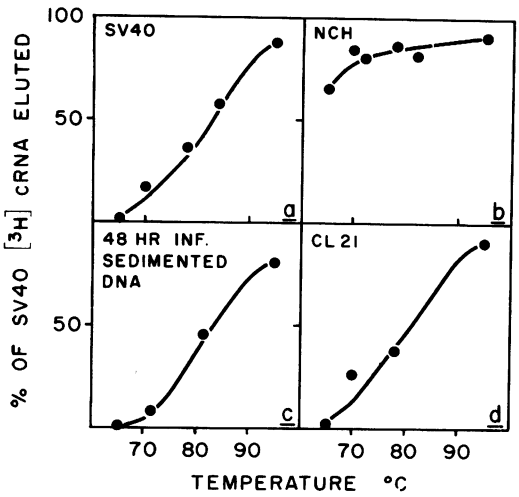

FIG. 7. *Thermal elution of SV40 ^{3}H-cRNA from DNA-RNA hybrids. DNA-RNA hybridization was done as described before. The input counts of SV40 ^{3}H-cRNA were 1.27 × 10^5 counts/min in 6× SSC containing 0.1 SDS. The amounts of immobilized DNA on the filter were: (a) SV40 DNA, 0.02 μg; (b) ChH DNA, 110 μg; (c) sedimented DNA from nuclei at 48 hr postinfection, 136 μg; (d) Cl-21 (SV40-transformed ChH cell line), 105 μg. After ribonuclease treatment, the filters were washed with 2× SSC, put into a vial containing 1 ml of 2 × SSC, and incubated for 10 min at the indicated temperature. The eluted radioactivity from the filter was counted. The percentage of the eluted radioactivity from the filter in (c) and (d) was corrected by subtraction of the radioactivity eluted from the [ChH-DNA + SV40-cRNA] hybrid (b). The total amounts of hybridized radioactivity on a filter before thermal elution were: (a) 3,204 counts/min, (b) 620 counts/min, (c) 2,236 counts/min, and (d) 1,452 counts/min.*

infected ChH DNA plus SV40-cRNA) hybrids but very different from that of the (SV40-cRNA plus ChH-DNA) hybrids. Thus, long complementary nucleotide sequences (14) are involved in the hybrids between the SV40 cRNA and the cellular DNA from infected cells at 48 hr postinfection as well as in the transformed C1-21 cells.

DISCUSSION

These experiments indicate that incoming SV40 DNA becomes associated with the DNA of ChH cells shortly after infection. Evidence for this association is derived from the presence of DNA sequences which hybridize with SV40 cRNA in the sedimented nuclear DNA isolated by the Hirt extraction method and in the high-molecular-weight DNA (>100S) sedimented in alkaline sucrose gradient.

The possibility that the amount of SV40 DNA found in the fast-sedimenting DNA of the nuclei from infected cells could be the result of contamination can be excluded on the basis of the following facts. In reconstruction experiments with various combinations of SV40 DNA and numbers of nuclei, at the most only 3% of the input viral DNA was found in the cell DNA fractions, whereas in gradients of nuclei from infected cells, up to 90% of DNA hybridizable with SV40 cRNA was observed in such fractions. Most importantly, the appearance of SV40 DNA covalently linked to cell DNA follows a clear chronological pattern, with none being demonstrable at 4 hr postinfection and a large amount being demonstrable at 30 hr postinfection. The situation would be different if circular polymers of the SV40 DNA, which would cosediment with cellular DNA, occurred. However, the formation of circular dimers or trimers has been observed only in 3T3 cells which were transformed by a thermosensitive mutant of polyoma virus and induced to produce virus by a shift to the permissive temperature (4). There is no evidence that such polymers may form during the early phases of virus infection before viral DNA replication.

The character of the association between SV40 DNA and cellular DNA described here is similar to that observed in SV40-transformed cells many cell generations after transformation has occurred (17), not only in having alkaline-stable linkages but also in the thermal stability of the DNA plus SV40 cRNA hybrids. Thus, by current definition, the SV40 DNA, or a substantial portion of it, appears to be integrated in the DNA of primary infected cells. The maximum level of integration, after infection at a given multiplicity, is reached by 30 hr postinfection and occurs during the first cycle of cellular DNA synthesis. This level does not appear to increase thereafter, probably because free viral DNA is no longer present in the nucleus after this period.

The level of SV40 cRNA hybridizable with the DNA of primary infected cells is of the same order as that observed in some clones of ChH cells transformed by SV40 (8). It is not possible, however, to define whether this value in the primary infected cultures indicates that the integration occurs in most of the cells at a similar level or that a large number of genome equivalents become integrated in only a fraction of the cell population. For this reason also, it is not possible at present to determine whether integration occurs only in cells which eventually will have the transformed phenotype. A small but definite amount of SV40 integration has been detected in permissive CV-1 cells infected under selective experimental conditions (K. Hirai and V. Defendi, *in preparation*).

As in the case of adenovirus 12 integration into the DNA of hamster cells (6), integration of SV40 DNA into the DNA of ChH cells is not

inhibited by Ara-C. If SV40 can be integrated into the cellular DNA in the absence of DNA synthesis, then the process may be analogous to the recombination between bacteriophage DNA species in which breakage and reunion occurs between preexisting DNA species (9, 12, 15). Although cellular or viral DNA replication is not required for integration, some intermediary changes of the cellular DNA may be necessary, such as availability, or activation, of an endonuclease.

By genetic analysis, it has been shown that lambda phage integration in *E. coli* DNA is catalyzed by an active protein produced by the *int* gene (18). On the basis of the results reported here, in which inhibition of protein synthesis reduced, but did not suppress, the level of integration, and those of Doerfler on the integration of adenovirus 12 with hamster cell DNA (6), it appears that the "integrating protein," if it exists in this system, is not a newly synthesized protein. In the case of adenovirus 12, it has been shown that one of the viral proteins has an endonuclease function (2); however, no such evidence is available for SV40.

From the results of several experimental approaches, it appears that there is a good chronological, as well as quantitative, correlation between integration and induction of cellular DNA synthesis and T antigen. Furthermore, a *ts* mutant of SV40 which at the nonpermissive temperature fails to induce T antigen or abortive or complete transformation [even though cell penetration and virus uncoating, as determined by susceptibility to deoxyribonuclease, are unaltered (J. Robb and K. Huebner, *personal communication*)] also fails to become integrated (Hirai, Robb, and Defendi, *unpublished data*). However, a causal relationship among these different parameters of early virus infection can only be a matter of speculation. ChH DNA contains short sequences complementary to SV40 DNA (8); thus, if integration occurs at these specific sites and if these sites are, or relate to, the initiation points for cellular DNA synthesis, it is possible to understand the mechanism by which cellular DNA synthesis is stimulated or altered after SV40 infection (13). Further circumstantial evidence lends support to the working hypothesis that integration is the primary event which determines further expression of the cell-virus interaction, namely, that induction of T antigen and of cellular DNA synthesis, alteration of cell surface, and the loss of topo inhibition by topographical factors (1, 5, 7) are inhibited under conditions in which integration is not.

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