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Intracellular Forms of Adenovirus DNA

III. Integration of the DNA of Adenovirus Type 2 into Host DNA in Productively Infected Cells

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KB cells productively infected with human adenovirus type 2 contain an alkali-stable class of viral DNA sedimenting in a broad zone between 50 and 90*S* as compared to 34*S* for virion DNA. This type of DNA is characterized as viral by DNA-DNA hybridization. It is extremely sensitive to shear fragmentation. Extensive control experiments demonstrate that the fast-sedimenting viral DNA is not due to artifactual drag of viral DNA mechanically trapped in cellular DNA or to association of viral DNA with protein or RNA. Furthermore, the fast-sedimenting DNA is found after infection with multiplicities between 1 and 1,000 PFU/cell and from 6 to 8 h postinfection until very late in infection (24 h). Analysis in dye-buoyant density gradients eliminates the possibility that the fast-sedimenting viral DNA represents supercoiled circular molecules. Upon equilibrium centrifugation in alkaline CsCl density gradients, the fast-sedimenting viral DNA bands in a density stratum intermediate between that of cellular and viral DNA. In contrast, the 34*S* virion DNA isolated and treated in the same manner as the fast-sedimenting DNA cobands with viral marker DNA. After ultrasonic treatment of the fast-sedimenting viral DNA, it shifts to the density positions of viral DNA and to a lesser extent to that of cellular DNA. The evidence presented here demonstrates that the 50 to 90*S* viral DNA represents adenovirus DNA covalently integrated into cell DNA.

Adenovirus DNA replication presents a model system for the study of viral DNA replication and integration in mammalian cells in tissue culture. To understand the mechanism of viral DNA synthesis, it is necessary to investigate the physical structure and biological function of the intracellular forms of viral DNA.

Previous studies on adenovirus DNA replication have revealed various forms of viral DNA. KB cells productively infected with adenovirus type 2 (Ad2) contain a DNA-RNA complex possibly involved in the transcription of viral DNA (10). Several laboratories have concentrated on the characterization of a partly single-stranded form of intracellular viral DNA which may represent an intermediate in viral DNA replication (26, 29, 31; and G. B. Pearson, personal communication). This form was found both in vivo (31) and in a system using isolated nuclei (29). A complex of viral DNA in association with protein has been isolated, though its role in replication is not clear (25, 33).

Horwitz (17) has described short pieces of viral DNA in Ad2-infected KB cells. However, Ad2 DNA as small as Okazaki fragments (24) is not predominant. Short pieces of adenovirus DNA have also been found in a system using isolated nuclei (29).

This communication describes a fast-sedimenting, alkali-stable form of viral DNA. This fast-sedimenting DNA is found throughout the course of viral DNA synthesis, and most probably represents a form of viral DNA integrated with cell DNA. It is possible that the fast-sedimenting viral DNA also consists in part of concatemeric adenovirus DNA.

Parts of this work have been presented at the Meeting of the Federation of the American Societies for Experimental Biology, April 1972, at the Fifth Tumor Virus meeting in Cold Spring Harbor, N.Y., August, 1973, and at the NATO Advanced Study Institute on Tumor Virus-Host Cell Interactions in Monte Carlo, September 1973.

MATERIALS AND METHODS

Media and sera. The media used in this study were Eagle medium (12) and the modification of Eagle

medium for suspension cultures. In some experiments Eagle medium for suspension cultures containing one tenth the amount of amino acids prescribed for the regular formulation was used. Calf serum was purchased from the Grand Island Biological Co. or from Flow Laboratories.

Solutions. PBS is phosphate buffered saline (11), PBS-d is the same solution without Mg^{2+} and Ca^{2+}. TE is 0.01 M Tris-hydrochloride, pH 7.2 to 7.5, 0.001 M EDTA, and STE is 0.5% sodium dodecyl sulfate (SDS), 0.1 M Tris-hydrochloride, pH 7.5, 0.02 M EDTA. The alkaline sucrose solutions consist of 5 or 20% sucrose (Schwarz/Mann) in 0.7 M NaCl, 0.3 M NaOH, 0.005 M EDTA. The TM scintillation mixture consists of 5 g of 2,5-diphenyloxazole, 0.3 g of 2,2'-p-phenylene-bis-(5-phenyloxazole), 500 ml of toluene, and 500 ml of methanol (both I.T. Baker grade).

Cells. KB cells, CCL 17 were purchased from the American Type Culture Collection. Human embryonic kidney (HEK) cells used for plaque assays were obtained from Flow Laboratories. CV-1 cells were purchased from Flow Laboratories.

Virus. Human Ad2 was a gift of Wallace Rowe, National Institutes of Health, Bethesda, Md. Simian virus 40 (SV40) was kindly provided by Gerd Sauer, Heidelberg.

Assay for mycoplasma. All cell cultures used were checked at least biweekly for mycoplasma contamination. The assay procedures of Hayflick (16) were used with minor modifications. The KB monolayer cultures used for each of the experiments described in this report were found to be free of mycoplasma.

Radioactive isotopes. The following compounds were bought from The Radiochemical Centre, Amersham, England: ^{32}P-carrier-free phosphate; [6-^{3}H]thymidine, 25 to 30 Ci/mmol; [2-^{14}C]thymidine, 50 to 62 mCi/mmol; [^{14}C]formic acid, Na salt, 50 to 60 mCi/mmol; [^{14}C]protein hydrolysate (U), 57 mCi/matom. The [^{3}H]L-amino acid mixture, 1 mCi/ml, was purchased from New England Nuclear Corp., Boston, Mass.

Preparation and purification of Ad2. Methods used for the cultivation and purification of Ad2 were previously described (5, 6, 9). KB cells in suspension culture were inoculated at an MOI of 10 to 20 PFU per cell, and were harvested 30 to 36 h postinfection, when virus yields were optimal (Rosenwirth, Tjia, Westphal, and Doerfler, Virology, in press). All inocula of Ad2 used in this study were purified by two or three cycles of equilibrium centrifugation in CsCl density gradients. The CsCl used for virus purification was the analytical grade of Merck, Darmstadt, Germany.

Storage of CsCl-purified virus. Purified virus was stored in the solution in which it was purified: CsCl in 0.02 M Tris-hydrochloride, pH 8.0. The virus kept its infectivity for several months under these conditions.

Preparation of [^{3}H]thymidine- or [^{14}C]formate-labeled virus. This method was described previously (5, 6). To label Ad2 with ^{3}H-amino acids, KB cells were infected with Ad2 and kept for 9 to 15 h in Eagle medium for suspension cultures containing 10% calf serum. Then the cells were collected by centrifugation (Christ UJ IV KS centrifuge, 800 to 1,000 rpm for 15 min) and were resuspended in Eagle medium for suspension cultures containing one tenth the amino acid concentration and 10% calf serum. The arginine concentration in this medium was supplemented to the full strength. Simultaneously, a mixture of ^{3}H-labeled amino acids was added at a concentration of 1 μCi per ml of medium. The infected cells were harvested 40 to 44 h postinfection, and the virus was purified as previously described.

Viral marker DNA was isolated from purified Ad2 labeled with [^{3}H]thymidine or [^{14}C]formate as reported earlier (9). SV40 was propagated in CV-1 cells. The DNA was labeled with [^{3}H]thymidine and the viral DNA was isolated as described elsewhere (2). Cellular DNA was labeled by growing KB cells in the presence of 1 μCi of [^{32}P]phosphate per ml. The methods for the purification of labeled DNA (9) or ribosomal RNA (10) from KB cells have been published elsewhere. The Ad2-specific DNA-RNA complex (HP) was double-labeled with [^{3}H]uridine (20 μCi/ml) and [^{14}C]thymidine (0.5 μCi/ml) and was isolated from KB cells as described previously (10).

Inoculation of KB cells with Ad2. KB cells were grown in monolayers in 60-mm diameter Falcon plastic dishes containing Eagle medium with 10% calf serum (MEMC). When the cell number per plate reached 10^6 to 4×10^6, the cells were inoculated with CsCl-purified Ad2 at an MOI of 100 PFU/cell. Prior to inoculation the virus was dialyzed for 2 to 4 h against PBS and was diluted in PBS.

The calculation of the MOI was based on previous determinations (5) indicating that 1 optical density unit at 260 nm (OD_{260}) of purified virus corresponded to 10^{10} PFU as determined on monolayers of HEK cells.

After a 90-min adsorption period, the inoculum was removed, the cells were washed three times with PBS, and 5 ml of fresh MEMC was added to each plate. The infected cells were labeled by adding [^{3}H]thymidine at a concentration of 30 μCi/ml and with a specific activity of 25 to 30 Ci/mmol to the medium at various times after infection. Since high activities of [^{3}H]thymidine were used, less than 1 μg of DNA per gradient was sufficient for analysis.

In some of the experiments the cellular DNA was prelabeled by maintaining the cells for 2 to 3 days prior to infection in medium containing [^{14}C]thymidine (0.4 μCi/ml of medium). In these experiments the cells were washed three times with PBS immediately before inoculation.

Lysis of cells and analysis of intracellular DNA by zonal sedimentation in alkaline sucrose density gradients. This method has been described earlier (5). At the end of the labeling period, the infected cells were washed six times with 3 ml of PBS and once with 4 ml of PBS-d. The cells were then scraped off the plate with a silicone policeman, counted, and resuspended in PBS-d at a concentration of 10^6 cells/ml. The cells were then placed on top of an alkaline sucrose density gradient; 70,000 cells in a large SW27 nitrocellulose tube and 30,000 cells in an SW41 nitrocellulose tube. It proved critical that the cell number per centrifuge tube not exceed 10^5. At higher cell numbers the high viscosity of the cellular

DNA led to disturbances during fraction collection. Since the DNA content of a cell is about 1.7×10^{-5} μg (21), 70,000 cells contain 1.2 μg of DNA, and 30,000 cells contain 0.5 μg of DNA. The SW27 tube (SW41 tube) contained a 4-ml (1-ml) cushion of alkaline CsCl solution (CsCl in 0.7 M NaCl, 0.3 M NaOH, 0.005 M EDTA; ρ = 1.8 g/cm^3) on the bottom of the tube and 32 ml (10 ml) of a 5 to 20% alkaline sucrose density gradient. A 1-ml amount (0.33 ml) of 0.5 N NaOH, 0.01 M EDTA was layered on top of the gradient, and the intact cells were added to this layer to be lysed. When ^{14}C-labeled Ad2 marker DNA was used, it was added to the 1-ml (0.33 ml) top layer along with the cells. Lysis at 4 C was allowed to proceed for a period of 17 to 18 h prior to centrifugation unless otherwise indicated.

After completion of lysis, the samples were centrifuged in the SW27 or SW41 rotor at 4 C in the model L2-65B Spinco ultracentrifuge. After centrifugation, 34 to 40 1.1-ml (0.3 ml) fractions were collected by puncturing the tubes. Samples of each fraction (usually 0.2 to 0.3 ml) were counted directly with 5 to 7 ml of TM solution as described elsewhere (W. Doerfler, Anal. Biochem., submitted for publication) or were precipitated by trichloroacetic acid, and the precipitates were collected on glass fiber filters (GF/C). Radioactivity was determined in a Packard TriCarb Scintillation Spectrometer, model 3385. Very similar results were obtained whether samples were counted directly or after precipitation by trichloroacetic acid.

Analysis of intracellular DNA by zonal sedimentation in self-forming alkaline CsCl gradients. Self-forming alkaline CsCl gradients were used in some experiments (32). The gradients were prepared as follows. A 1-ml cushion of alkaline CsCl (see above) was overlaid with 10 ml of CsCl in 0.1 N NaOH, 0.05 M EDTA (ρ = 1.5 g/cm^3) in a nitrocellulose SW41 tube. On top of this solution 0.35 ml of 0.5 N NaOH, 0.01 M EDTA was layered. Approximately 30,000 Ad2-infected KB cells in 0.03 ml of PBS-d were lysed by placing them in this top layer, and a small amount of ^{14}C-labeled Ad2 DNA was added as marker. After 18 h of lysis at 4 C, centrifugation was carried out in the SW41 rotor at 35,000 rpm for 140 min at 4 C.

Analysis by equilibrium sedimentation in neutral CsCl gradients. The total intracellular DNA from Ad2-infected KB cells was isolated by STE-lysis of the cells and digestion of the extracts with Pronase B (250 μg/ml of extract). This extract was purified further with phenol (saturated with 1 M Tris-hydrochloride, pH 7.5), ether and N_2 (6). The DNA isolated in this manner was subsequently analyzed by equilibrium sedimentation in neutral CsCl density gradients. A 1-ml amount of extract was layered on top of 4 ml of a CsCl solution consisting of 15 g of CsCl and 10 ml of TE. Sedimentation was performed as described in the legend to Fig. 1.

Analysis by equilibrium sedimentation in alkaline CsCl density gradients. Pooled fractions from alkaline sucrose density gradients were analyzed by equilibrium sedimentation in alkaline CsCl gradients. The fractions from alkaline sucrose density gradients were neutralized by the addition of a predetermined amount of 1 M HCl and 1 M Tris-hydrochloride, pH 7.2. Alkaline CsCl density gradients were prepared by adding 0.8 ml of solution A to 3.2 ml of solution B. Solution A consists of 1.0 ml of pooled neutralized fast-sedimenting material from the alkaline sucrose gradients, 0.05 ml of 0.1 M EDTA, and 0.1 ml of 6 N NaOH. Solution B contains 1.5 ml of pooled neutralized fast-sedimenting material from the alkaline sucrose gradients, 1.5 ml of 0.1 M sodium phosphate buffer (pH 11.7), 0.02 M EDTA, 0.02 M NaOH, and 5.17 g of CsCl. In a control experiment, 34S viral DNA isolated from alkaline sucrose gradients was treated in the same manner as fast sedimenting DNA. The gradients were centrifuged to equilibrium in an SW56 rotor of the Spinco L2-65B ultracentrifuge at 36,000 rpm for 60 h at 20 C. Small amounts of ^{14}C-labeled Ad2 DNA and ^{32}P-labeled KB cellular DNA were added as density markers. At the end of the 60-h centrifugation period, three-drop fractions were collected directly on GF/C filters. The samples were precipitated on the filter with trichloroacetic acid, washed with acid and ethanol, dried, and counted.

Ultrasonic treatment of DNA. In some experiments the pooled fractions of DNA from alkaline sucrose density gradients were fragmented by ultrasonic treatment prior to equilibrium sedimentation in alkaline CsCl density gradients. The microtip of a Branson sonifier was used. The ultrasonic treatment lasted for 1 min at 55 W (position 2).

Equilibrium sedimentation in dye-buoyant density gradients by using CsCl propidium iodide. The method of Hudson et al. (18) was used as described earlier (9).

Resedimentation of the fast-sedimenting DNA. Since the fast-sedimenting DNA is very sensitive to shear breakage, pipetting had to be avoided. Therefore, fractions containing fast-sedimenting DNA were pooled for resedimentation and poured onto a 15 to 30% alkaline sucrose density gradient.

Polyacrylamide gel electrophoresis of proteins. Incompletely digested protein associated with the fast-sedimenting DNA was analyzed by using polyacrylamide gel electrophoresis. The method for preparing polyacrylamide gels was described by Everitt et al. (14). Acrylamide and *N*,*N*'-methylenebisacrylamide were recrystallized as described earlier (10). To prepare the fast-sedimenting DNA and associated protein for electrophoresis, the samples containing the protein were first pooled and then dialyzed against 0.02% SDS, 0.0001 M Tris-hydrochloride, pH 7.2. The dialysate was then concentrated by lyophilization and resuspended in distilled water before electrophoresis. It was necessary to include SDS in the dialysis buffer to prevent heavy losses of material. The ^{14}C-labeled samples and ^{3}H-amino acid-labeled Ad2 marker virus were electrophoresed at 100 V., 8 mA for 5.5 h. Subsequently, the gels were sliced and processed for scintillation counting as reported earlier (30).

Isolation of nuclei from Ad2-infected KB cells. To isolate nuclei from Ad2-infected KB cells, the cells were first washed extensively in PBS and PBS-d and then suspended in isotonic buffer, 0.15 M NaCl, 0.015 M Tris-hydrochloride, pH 7.5, 0.0015 M $MgCl_2$, 0.5% NP-40 (nonionic detergent, Shell Oil Co.). They were

then stirred by a Vortex mixer, and subsequently incubated at 4 C for 15 min. Released nuclei were pelleted by low-speed centrifugation, washed once in the buffer described above, and then suspended in 0.15 M NaCl, 0.015 M Tris-hydrochloride, pH 7.5, 0.0015 M $MgCl_2$ at a concentration of approximately 10^6 nuclei/ml. About 30,000 nuclei were then lysed by layering them in alkali on top of an alkaline sucrose gradient.

DNA-DNA hybridization was performed according to the method of Denhardt (3) as described previously (6). Samples from alkaline sucrose density gradients or from CsCl gradients were neutralized, diluted 1:1 with water, sonically treated, heat-denatured (100 C for 5 to 10 min), and then added directly to the Denhardt mixture for hybridization. Samples equal in size to those taken for hybridization were precipitated with trichloroacetic acid and counted to determine the input radioactivity.

Plaque assay. Titers of Ad2 preparations were determined by plaquing on HEK cells in secondary passage by the method described earlier (22).

Data analysis. The Liquid Scintillation Spectrometer data were processed via Teletype tape in a Wang 720 B calculator equipped with a tape recorder as described elsewhere (W. Doerfler, Anal. Biochem., submitted for publication).

All other methods for physical and chemical measurements have been described previously (5, 6, 9, 10).

RESULTS

Time course of viral DNA synthesis. Ad2 DNA can be separated from KB cell DNA by equilibrium sedimentation in neutral CsCl gradients (5, 13). This separation is shown in a reconstitution experiment illustrated in Fig. 1 and can be used to determine the type of DNA synthesized in Ad2-infected KB cells at various times after infection. Viral DNA synthesis is detected as early as between 6 and 8 h postinfection (Fig. 1). The bulk of the DNA synthesized early after infection is cellular, since nonconfluent cultures of KB cells were used in this experiment. There is an increase in the amount of newly synthesized viral DNA with time and eventually viral DNA becomes the predominant species (12 to 14 h postinfection). Figure 1 shows that between 14 to 16 and 16 to 18 h postinfection, DNA with the buoyant density of Ad2 DNA is the main species synthesized. In the uninfected control only cellular DNA is detectable. However, even late after infection (22 to 24 h postinfection, not shown in graph) there remains a low level of [^{3}H]thymidine incorporation into DNA in the density stratum of cellular DNA. In most experiments described in this report [^{3}H]thymidine is added from 14 to 17 h postinfection, when most of the newly synthesized DNA is viral.

Newly synthesized DNA centrifuged to equilibrium in neutral CsCl density gradients bands in three main density strata: viral (ρ = 1.715 g $\times$ cm^{-3} [5]), intermediate, and cellular (ρ = 1.699 g per cm^{-3} [5]). DNA from each of these strata has been analyzed by DNA-DNA hybridization to viral DNA. DNA in the viral density positions hybridizes as expected. In addition, DNA in the intermediate and cellular positions shows surprisingly high levels of hybridization as well. This hybridization of intermediate and cellular DNA to viral DNA is first seen 12 to 14 h postinfection. By 14 to 16 h and 16 to 18 h postinfection, almost all of the labeled DNA in these density positions hybridizes with viral DNA. Upon resedimentation, approximately 30 to 50% of the ^{3}H-labeled DNA in the intermediate and cellular density positions bands in the same density strata. Thus, it may represent newly synthesized Ad2 DNA associated with cellular DNA.

Fast-sedimenting, alkali-stable DNA in Ad2-infected KB cells. In earlier work (Fig. 4 of ref. 1), fast-sedimenting, alkali-stable DNA was found in Ad2-infected KB cells; however, only transiently and as a minor component. It now appears that only small amounts of fast-sedimenting DNA were seen because of the procedure used to extract DNA at that time. This procedure consisted of extraction by SDS lysis, Pronase treatment of the lysate, and phenolization, a procedure that could easily have fragmented high molecular weight DNA. To avoid fragmentation, a more gentle method of extraction of the intracellular DNA has been applied (5, 20, 23), lysis of intact cells or nuclei in alkali. The results presented in Fig. 2a demonstrate that after direct lysis of the Ad2-infected cells in 0.5 M NaOH, four size-classes of DNA can be separated by zonal sedimentation in alkaline sucrose density gradients:

(i) DNA sedimenting very rapidly on to the cushion of alkaline CsCl on the bottom of the gradient. This class of DNA contains most of the ^{14}C-prelabel and represents predominantly cellular DNA. The apparent variation in the amount of this class of DNA (cf. Fig. 2a, b, and 3) may be explained by differences in the number of cells per plate. The amount of cellular DNA synthesized in each experiment will depend on the number of cells per plate.

(ii) DNA sedimenting in a broad region between 50 and 90*S*. This DNA contains small yet varying amounts of prelabel, although some experiments, such as those presented in Fig. 3a and 5, show more prelabel than seen in the experiment presented in Fig. 2a.

(iii) The main peak of ^{3}H-labeled DNA is free of ^{14}C-prelabel and co-sediments at 34*S* (7) with ^{14}C-labeled Ad2 marker DNA extracted

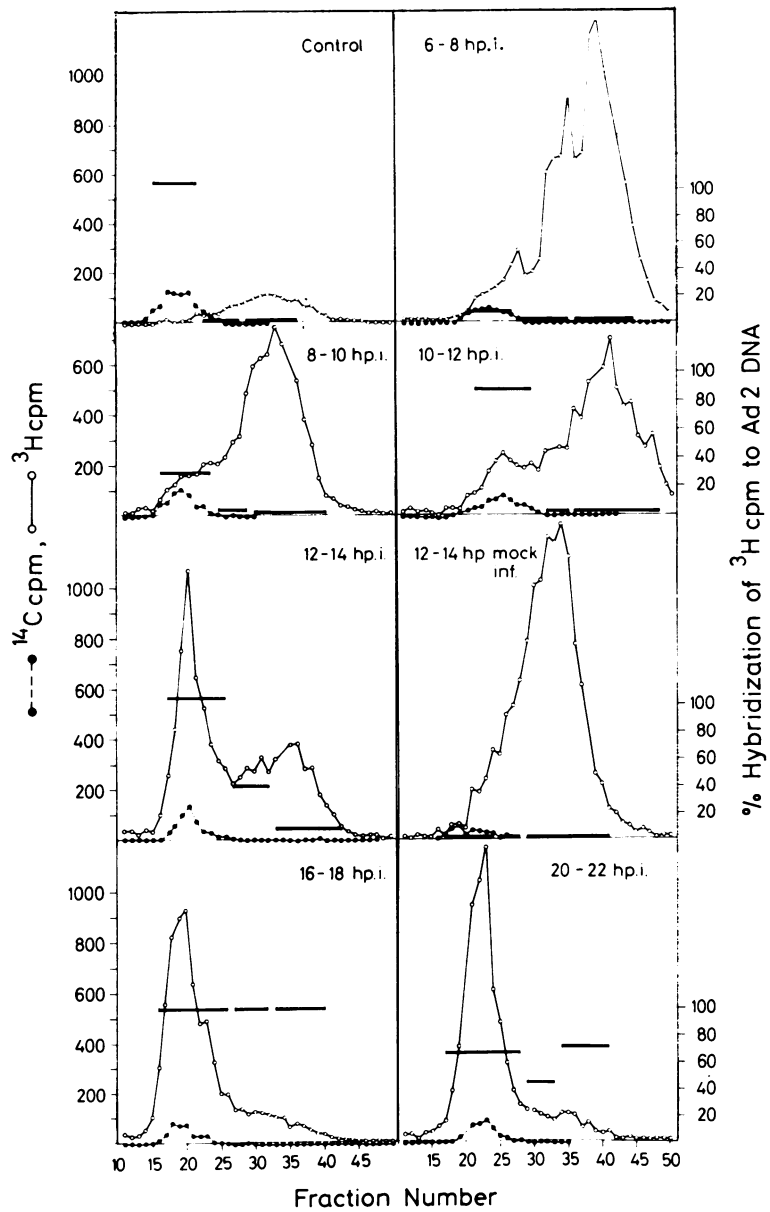
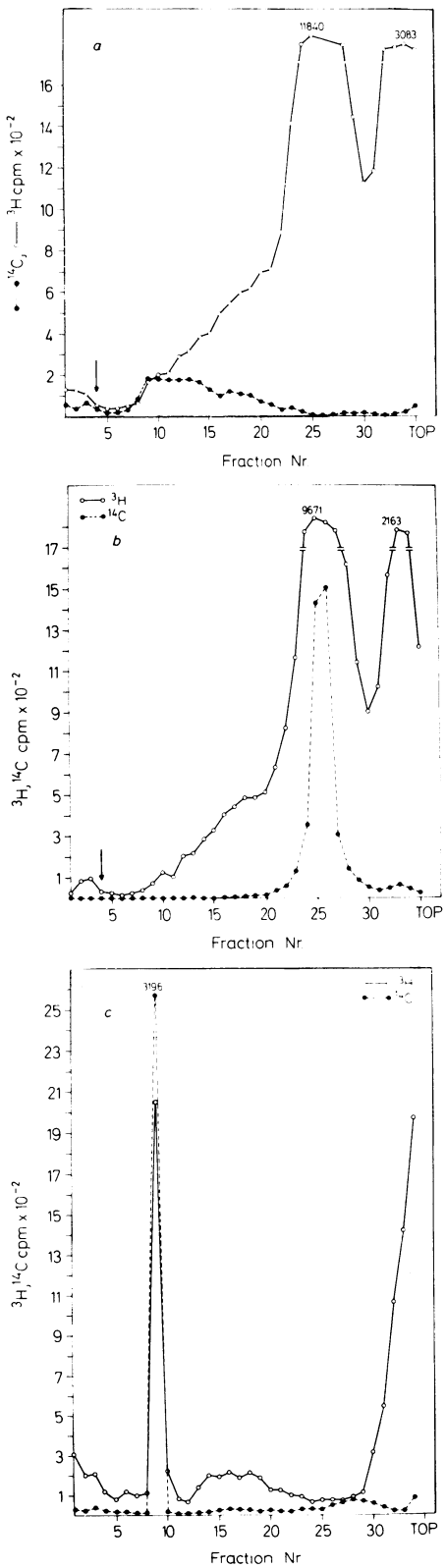


FIG. 1. *Time course of viral DNA synthesis in Ad2-infected KB cells. Analysis of the newly synthesized DNA by equilibrium sedimentation in neutral CsCl density gradients. KB cells growing in monolayers were infected with unlabeled, CsCl-purified Ad2 at an MOI of 100 PFU/cell or were mock-infected with PBS. At various times after infection, as indicated in the graph, the infected cells were labeled by adding 30 μCi of [^3H]thymidine per ml to the medium. At the end of the 2-h labeling period, the medium was removed, the cells were extensively washed with PBS and then lysed, and the total intracellular nucleic acid was extracted. The total intracellular nucleic acid was analyzed by equilibrium sedimentation in neutral CsCl density gradients (see Materials and Methods) by using the 50 Ti fixed angle rotor of the L2-65B Spinco ultracentrifuge. To each tube ^{14}C-labeled Ad2 DNA was added as density marker. The samples were centrifuged at 33,000 rpm for 59 h at 20 C. At the end of the centrifuge run, approximately 80 three-drop fractions were collected from the bottom of the tube. In 10-μliter portions of each fraction the ^3H- and ^{14}C-activities were determined. Fractions from each gradient were pooled as indicated by the lengths of the horizontal bars. The DNA in each of these pools was hybridized to Ad2 DNA by the filter method. The height of the horizontal bars in the graphs indicates the percentage of the ^3H-activity that hybridizes to Ad2 DNA. In the panel designated control, the height of the horizontal bars indicates the percentage of the ^{14}C-activity hybridizing to Ad2 DNA. In the control experiment a mixture of ^3H-labeled KB DNA and ^{14}C-labeled Ad2 DNA was centrifuged to equilibrium. Density increases to the left in all graphs. On the abscissa the scale begins with fraction 10. There is no radioactivity found in fractions 1 to 10.*



from purified virions (Fig. 2b). It should be emphasized that the reference DNA sediments as a sharp, symmetrical peak demonstrating that 34*S* viral DNA is not dragged into faster sedimenting regions.

(iv) Slowly sedimenting DNA probably representing short pieces of viral DNA. The nature of this material has not been studied in this report.

The present study deals with the characterization of the 50 to 90*S* DNA. When mock-infected cells prelabeled with [^{14}C]thymidine are analyzed in the same way (Fig. 2c), the bulk of the ^{3}H-label is found on top of the cushion together with the mass of ^{14}C-prelabeled cellular DNA. There is a minor peak heterogeneous in size, approximately corresponding to the 50 to 90*S* region in the gradient. Thus, this size-class of DNA is found both in uninfected and Ad2-infected KB cells.

DNA in the 50 to 90*S* size class has been observed in KB cells after Ad2 infection at

FIG. 2. *DNA synthesis in Ad2-infected KB cells. Analysis by zonal sedimentation in alkaline sucrose density gradients. KB cells growing in monolayers were infected with unlabeled CsCl-purified Ad2 at an MOI of 100 PFU/ml or were mock-infected with PBS. In the experiments illustrated in parts a and c the KB cells were prelabeled by adding [^{14}C]thymidine (0.4 μCi/ml) to the medium and maintaining the cells for 3 days prior to infection under these conditions. In the experiment shown in part b the cells were not prelabeled. In experiments a and c the cells were washed several times with PBS immediately prior to infection, to remove the ^{14}C-prelabel. At various times after infection, the newly synthesized DNA was labeled with [^{3}H]thymidine (30 μCi per ml of medium). At the end of the labeling period, the cells were washed several times with PBS and were lysed for 18 h on top of an alkaline sucrose gradient. (a) KB cells prelabeled with [^{14}C]thymidine were infected with Ad2 and the newly synthesized DNA was labeled with [^{3}H]thymidine from 14 to 17 h postinfection. Centrifugation was at 22,000 rpm for 380 min at 4 C in the SW27 rotor of the L2-65B ultracentrifuge. The ^{3}H-counts per minute values of fractions 24, 26, 27, 28, 32, 33, and 35, not drawn to scale are 3,125, 10,851, 3,475, 2,174, 1,926, 2,586, and 1,817, respectively. (b) KB cells were infected with Ad2 and the newly synthesized DNA was labeled with [^{3}H]thymidine from 14 to 17 h postinfection. The sample was lysed in alkali for 18 h together with ^{14}C-labeled Ad2 marker DNA and then centrifuged in the SW41 rotor at 35,000 rpm for 140 min at 4 C. The ^{3}H-counts per minute values of fractions 24, 26, 27, and 34 not drawn to scale are 2,806, 9,173, 2,838, and 1,992, respectively. (c) KB cells prelabeled with [^{14}C]thymidine were mock-infected with PBS and labeled with [^{3}H]thymidine from 16 to 18 h after mock-infection. The cells were lysed in alkali and centrifuged at 23,000 rpm for 430 min at 4 C in the SW 27 rotor. The arrows indicate the height of the CsCl shelves.*

multiplicities of 1, 10, 100, and 1,000 PFU/cell. The results listed in Table 1 demonstrate that with increasing multiplicities there is a decrease in the relative amount of fast-sedimenting DNA. This decrease may reflect the degree to which cellular DNA synthesis is progressively inhibited at increasing multiplicities. The relative amount of labeled DNA in the 50 to 90S region ranges from 16.9 to 56.5% of the total label incorporated.

The 50 to 90S DNA is observed in experiments in which the labeling period with [^{3}H]thymidine was as brief as 30 min. In short pulses (1 to 7 min) the 50 to 90S peak is not evident.

The 50 to 90S DNA is detected from 8 to 10 h postinfection and is seen throughout the course of infection.

Characterization of the 50 to 90S DNA by DNA-DNA hybridization. Fast-sedimenting DNA is present in both uninfected and Ad2-infected cells. To determine if the fast-sedimenting DNA from infected cells contains viral sequences, it is necessary to hybridize it to viral DNA. In the experiment shown in Fig. 3a, ^{14}C-prelabeled cells were infected with Ad2, were labeled with [^{3}H]thymidine from 14 to 17 h postinfection, and were analyzed by zonal sedimentation. After centrifugation, samples of every other fraction were hybridized to Ad2 and to cellular DNA. The data demonstrate that the 50 to 90S DNA and the 34S peak hybridize to viral DNA (Fig. 3b). Therefore, the fast-sedimenting 50 to 90S DNA is at least partly viral in nature. Hybridization to cellular DNA is not detectable by the filter hybridization technique. A more detailed analysis measuring the reassociation kinetics of the fast-sedimenting DNA with Ad2 and cellular DNA is in progress. Preliminary results (Burger, Fanning, and Doerfler, unpublished work) show that relatively large amounts of viral DNA are detectable in the 50 to 90S region by reassociation measurements.

TABLE 1. *Distribution of ^{3}H-radioactivity among fast-sedimenting DNA, 34S viral DNA, and fragmented DNA in alkaline sucrose density gradients*[a]

MOI (PFU/cell)	^{3}H-activity in					
	Fast-sedimenting region		Viral DNA peak		Fragments	
	Counts/min	Total (%)	Counts/min	Total (%)	Counts/min	Total (%)
1	14,197	56.5	6,459	25.7	4,483	17.8
10	15,227	47.6	12,158	38.0	4,585	14.3
100	1,748	28.2	2,611	42.2	1,820	29.4
1,000	3,305	16.9	11,837	60.7	4,366	22.3

[a] KB cells growing in suspension culture were infected with Ad2 at multiplicities as indicated. The cells were labeled with [^{3}H]thymidine (30 μCi/ml) between 14.5 and 17.5 h postinfection and were then lysed and analyzed as described in the legend to Fig. 2b. The total ^{3}H-activity was computed in three regions of the gradient: the fast-sedimenting region (fractions 7 to 20 in Fig. 2b); the region of the peak of viral DNA (fractions 21 to 30 in Fig. 2b); and the more slowly sedimenting region (fractions 31 to 35 in Fig. 2b). The data presented in this Table were not actually taken from the experiment shown in Fig. 2b, but from a similar set of experiments.

Resedimentation of fast-sedimenting viral DNA. The fast-sedimenting viral DNA can be isolated from an alkaline sucrose density gradient and can be recentrifuged. It resediments at approximately 50 to 90S (Fig. 4). To achieve resedimentation at this rate, it is necessary to avoid shear breakage, pipetting, and dialysis. The resedimentation at 50 to 90S establishes the fast-sedimenting DNA as a real and distinct class of viral DNA.

Fast-sedimenting DNA is not an artifact of the lysis or sedimentation procedure. When an alkaline sucrose density gradient is "overloaded" by lysing 200,000 cells in alkali in an SW41 tube instead of the usual 30,000 cells lysed in a "regular" experiment, the same sedimentation pattern both of the prelabeled and the newly synthesized DNA profiles is obtained (Fig. 5).

It is highly unlikely that the fast-sedimenting DNA is produced as an artifact of excessive amounts of DNA on gradients.

In the zonal sedimentation experiments described in this report the amount of DNA per gradient was 1 μg or less. As mentioned in Materials and Methods, 30,000 cells correspond to approximately 0.5 μg of DNA (21). In one experiment an alkaline sucrose density gradient was "under-loaded" with approximately 8,000 labeled, Ad2-infected cells (about 0.14 μg of DNA). Upon zonal sedimentation, the 50 to 90S DNA was observed in the same relative amount as in the experiments with 30,000 or 70,000 cells per gradient. Thus, there is no effect on the relative amount of fast-sedimenting DNA when the number of cells lysed in a gradient varies by a factor of 25.

It is possible that the fast-sedimenting viral DNA results from incomplete dissociation of viral DNA from viral coat proteins (e.g., core proteins) or their precursors. However, when purified ^{3}H-amino acid-labeled Ad2 virus is lysed in alkali for only 90 min and then centrifuged, all the ^{3}H-label remains at the top of the alkaline sucrose density gradient. To simu-

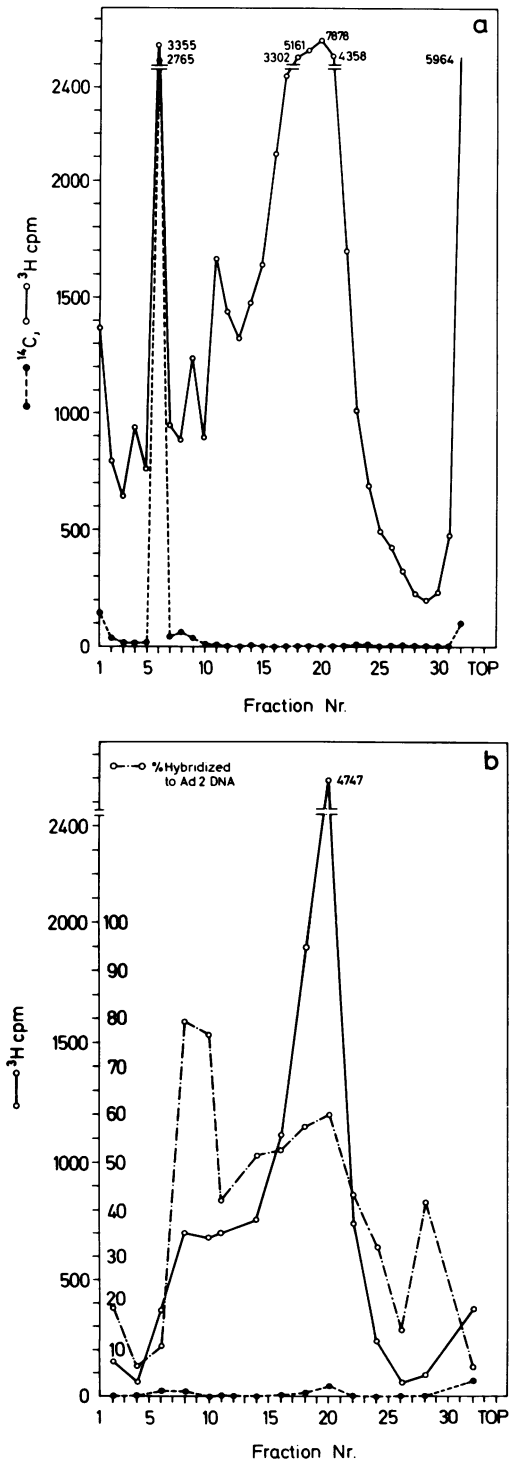


FIG. 3. *Characterization of fast-sedimenting DNA by DNA-DNA hybridization. (a) KB cells growing in monolayers were labeled with [^{14}C]thymidine (0.4 μCi/ml) for 4 days. The medium was then removed, the cells were carefully washed with PBS and were infected with Ad2 at an MOI of 100 PFU/cell. The cells were labeled with 50 μCi of [^{3}H]thymidine per ml of medium from 14 to 17 h postinfection. At the end of the labeling period, the cells were washed with PBS, scraped off the plastic dish, and lysed on top of an alkaline sucrose gradient. The sample was centrifuged in the SW27 rotor at 23,000 rpm for 7 h at 4 C. Fractions were collected; 0.2-ml samples were acid precipitated and analyzed. (b) The ^{3}H- and ^{14}C-labeled DNA molecules in the fractions from the experiment described in (a) were characterized by DNA-DNA hybridization. A 5-μg amount of unlabeled Ad2 DNA or 5 μg of unlabeled KB DNA was fixed to nitrocellulose filters. Every other fraction was analyzed. Samples of 0.2 ml were taken for hybridization. The samples were identical in volume to those taken in section (a). Symbols: O——O, ^{3}H-counts per minute hybridized to Ad2 DNA; ●, ^{3}H-counts per minute hybridized to KB DNA; O-·-·-·-·-O percentage of ^{3}H-counts per minute hybridized to Ad2 DNA.*

late conditions of the actual experiments in this control, the ^{3}H-amino acid-labeled virus was mixed with approximately 30,000 unlabeled Ad2-infected KB cells prior to lysis. Viral protein was removed in only 90 min of alkali lysis in this control. However, in almost all experiments performed in this study, alkali lysis was extended for 17 to 18 h at 4 C. Therefore, it is very unlikely that virion or cellular proteins could still be associated with viral DNA after alkali lysis (compare results presented in Fig. 9).

In a similar control experiment a sample of [^{3}H]thymidine-labeled, purified Ad2 was mixed with ^{14}C-labeled Ad2 marker DNA and about 30,000 unlabeled, Ad2-infected KB cells. After 18 h of lysis at 4 C in 0.5 N NaOH, the DNA was sedimented. Both ^{3}H-viral DNA and reference DNA co-sediment in one sharp, symmetrical peak. There is no evidence for fast-sedimenting DNA (Fig. 6). This datum argues strongly against artifactual drag as an explanation for the occurrence of fast-sedimenting viral DNA.

Analysis of [^{3}H]thymidine-labeled, Ad2-infected KB cells by zonal sedimentation in self-forming, alkaline CsCl gradients (32) yields results identical to those obtained in alkaline sucrose density gradients (Fig. 7). Thus, in CsCl solution of high ionic strength (ρ = 1.50 g/cm^{3}) an identical sedimentation pattern is obtained as in sucrose density gradients.

It was necessary to determine whether cytoplasmic factors play an artifactual role in generating fast-sedimenting viral DNA. Nuclei were isolated from Ad2-infected KB cells as described in Materials and Methods and about 30,000 nuclei were lysed in alkali and sedi-

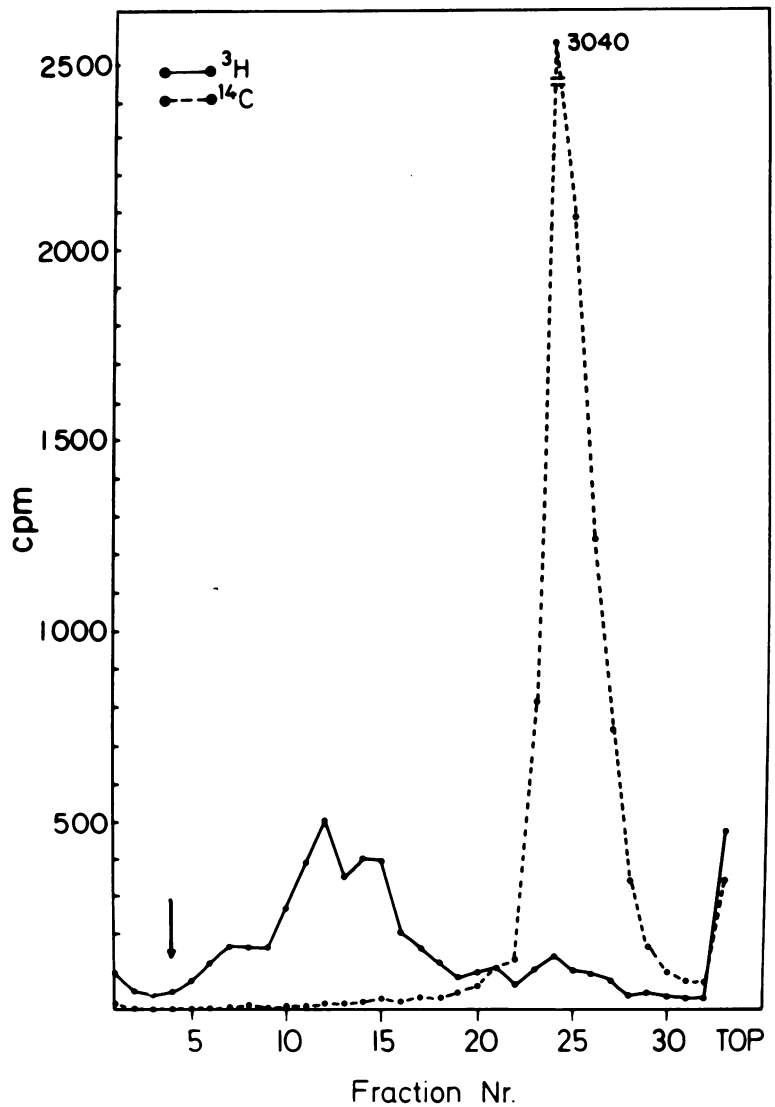


FIG. 4. *Resedimentation of fast-sedimenting viral DNA in alkaline sucrose density gradient. Fast-sedimenting viral DNA was isolated in an experiment similar to the ones described in Fig. 2a,b, and 3a. The fractions comprising the fast-sedimenting peak were carefully poured on top of an alkaline sucrose density gradient (15 to 30%) together with ^{14}C-labeled Ad2 marker DNA. Pipetting of the samples was avoided to minimize shear breakage of the fast-sedimenting DNA. Conditions of sedimentation: SW27 rotor, 23,000 rpm for 10 h at 4 C. Fractions were collected and analyzed. The DNA in fractions 8 to 16 was calculated to have approximate S values of 50 to 90. The arrow indicates the height of the CsCl shelf.*

mented. The result of this sedimentation (Fig. 8) is similar to that obtained when intact cells are used, thus showing that cytoplasmic factors are not likely to be responsible for fast-sedimenting viral DNA.

To determine if the fast-sedimenting DNA could be a complex of RNA and viral DNA, a sample of ^{14}C-labeled ribosomal RNA from KB cells was lysed in alkali for 18 h at 4 C as described above. After sedimentation, all the radioactivity was found on top of the gradient, showing that under the conditions used in this experiment RNA is hydrolyzed and thus cannot account for the high sedimentation rate of the 50 to 90S viral DNA.

It might be argued that Ad2-specific mRNA, particularly in a complex with viral DNA, could be resistant to alkali digestion at 4 C. This possibility is eliminated by studies on an Ad2-specific DNA-RNA complex which have been

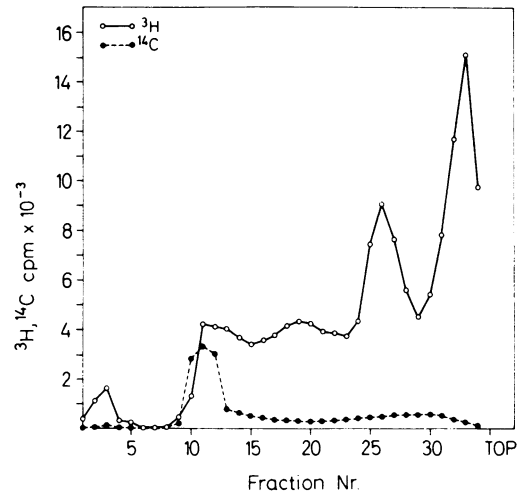


FIG. 5. *"Overloading" the gradient with DNA does not alter result of sedimentation experiment. KB cells growing in monolayers were prelabeled with [^{14}C]thymidine (0.4 μCi/ml), were infected with CsCl-purified Ad2 at an MOI of 100 PFU/cell and were labeled with [^{3}H]thymidine (30 μCi/ml) from 14 to 17 h postinfection. Instead of 70,000 cells being lysed on top of an alkaline sucrose density gradient in an SW27 rotor, 200,000 cells were lysed on top of an alkaline sucrose density gradient in an SW41 rotor, Conditions of centrifugation: 37,000 rpm at 4 C for 133 min. At the end of the centrifuge run, 12-drop fractions were collected.*

described previously (10). In addition, an even more rigorous control has been performed. The DNA-RNA complex from Ad2-infected KB cells was labeled with [^{14}C]thymidine and [^{3}H]uridine and was isolated as described earlier (10). The complex was then treated with 0.5 N NaOH, 0.05 M EDTA on top of an alkaline sucrose gradient at 4 C for 20 h. After zonal sedimentation, all the ^{3}H-label remained on top of the gradient. These experiments rule out the possibility that the fast-sedimenting viral DNA represents a DNA-RNA complex.

Digestion of protein during alkali lysis. The data in Fig. 9a and b demonstrate that it is essential to lyse the Ad2-infected cells in 0.5 N NaOH at 4 C for many hours to remove protein efficiently from fast-sedimenting viral DNA. After short incubation periods (1 h), appreciable amounts of protein remain associated with the fast-sedimenting DNA (Fig. 9a). Preliminary analysis of this protein moiety by SDS-polyacrylamide gel electrophoresis suggests that it is homogeneous; however, its nature is unknown. In all experiments described here, a 17- to 18-h incubation period in alkali has been used. Since the protein is effectively removed from the fast-sedimenting DNA by long incubation in alkali, aggregation with protein cannot explain the high sedimentation rate.

Analysis of the fast-sedimenting viral DNA in dye-buoyant density gradients. It is conceivable that the fast-sedimenting viral DNA represents supercoiled circular molecules. This possibility has been eliminated by equilibrium sedimentation in dye-buoyant density gradients. When a mixture of ^{14}C-labeled Ad2 marker DNA and ^{3}H-labeled fast-sedimenting viral DNA was denatured in alkali, neutralized, and sedimented to equilibrium in dye-buoyant density gradients, the fast-sedimenting DNA cobands exactly with the Ad2 marker (Fig. 10a and b). Therefore, the fast-sedimenting DNA cannot contain supercoiled stretches. It has previously been shown (10) that supercoiled, covalently closed viral DNA cannot be isolated from Ad2-infected KB cells.

To demonstrate that supercoiled, covalently closed DNA is resistant to extensive alkali treatment, a mixture of ^{3}H-labeled SV40 forms I and II DNA has been incubated in 0.5 N NaOH for 19.5 h at 4 C, neutralized, and centrifuged to

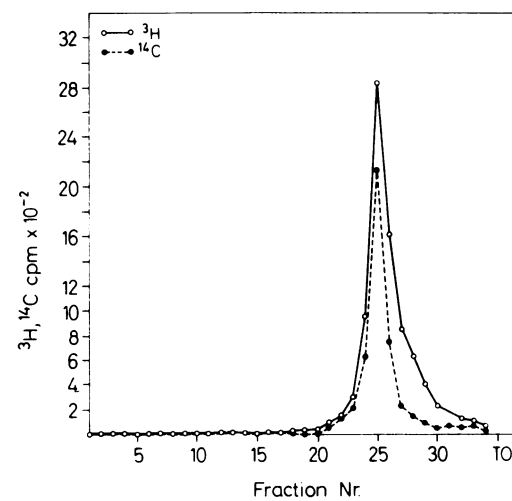


FIG. 6. *Control experiment: sedimentation analysis of [^{3}H]thymidine-labeled Ad2 virus in alkaline sucrose density gradients. KB cells growing in monolayers were infected with unlabeled Ad2 at an MOI of 100 PFU/cell and were washed and lysed in alkali on top of an alkaline sucrose density gradient. Immediately prior to lysis, the cells were mixed with a sample of [^{3}H]thymidine-labeled Ad2 virus purified in CsCl density gradients. A sample of ^{14}C-labeled Ad2 DNA was added to the alkaline mixture as marker. The sample was kept at 4 C for 90 min on top of an alkaline gradient prior to centrifugation in an SW41 rotor. Conditions of centrifugation: 35,000 rpm at 4 C for 140 min; 12-drop fractions were collected after centrifugation.*

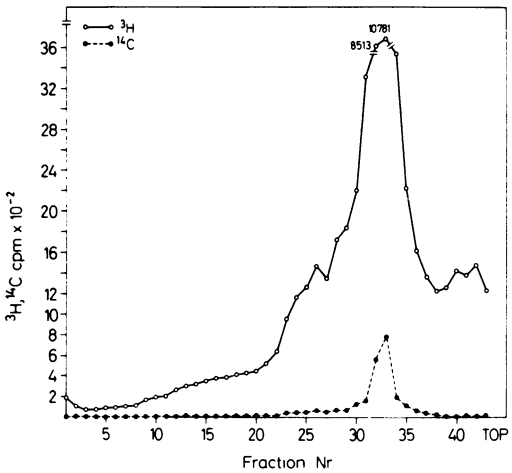


FIG. 7. *Fast-sedimenting viral DNA: analysis by zone sedimentation in an alkaline CsCl gradient. KB cells growing in monolayers were infected with CsCl-purified Ad2 at an MOI of 100 PFU/ml. The infected cells were labeled with [^3H]thymidine (30 μCi/ml) from 14 to 17 h postinfection. At the end of the labeling period, the cells were washed and approximately 30,000 cells were lysed in alkali on top of an alkaline CsCl gradient. A sample of ^{14}C-labeled Ad2 DNA was added as marker. After 18 h of incubation at 4 C, the sample was centrifuged in an SW41 rotor at 35,000 rpm for 140 min at 4 C. After centrifugation, 12-drop fractions were collected and analyzed.*

equilibrium in dye-buoyant density gradients (Fig. 11a). A control sample has been kept in 0.01 M Tris-hydrochloride at neutral pH and otherwise has been treated identically (Fig. 11b). The results of this experiment show that SV40 form I DNA is stable in alkali and can still be separated from linear DNA in dye-buoyant density gradients even after extensive alkali treatment. Furthermore, it is possible to isolate SV40 form I DNA from SV40-infected CV-1 cells by lysing the cells in 0.5 N NaOH and sedimenting the viral DNA in alkaline sucrose density gradients (W. Doerfler, unpublished results). These data conclusively demonstrate that supercoiled circular DNA is stable in 0.5 N NaOH at 4 C for 18 to 20 h and can still be isolated in dye-buoyant density gradients. Thus, the finding that 50 to 90S Ad2 DNA co-bands with linear marker DNA in dye-buoyant density gradients (Fig. 10) demonstrates that the fast-sedimenting DNA does not contain covalently closed, supercoiled molecules.

Analysis of the 50 to 90S DNA and the 34S DNA by equilibrium sedimentation in alkaline CsCl density gradients. The experimental evidence presented so far eliminates the possibilities that the alkali-stable, fast-sedimenting Ad2 DNA represents supercoiled circular DNA molecules or viral DNA associated with protein or RNA. It is possible to determine whether the fast-sedimenting DNA consists of a concatemeric form of Ad2 DNA or a form of Ad2 DNA covalently integrated into cellular DNA by determining the buoyant density of the fast-sedimenting DNA in alkaline CsCl density gradients. The DNA of Ad2 and KB cells can be separated by equilibrium sedimentation in alkaline CsCl density gradients as seen by the positions of the ^{14}C-labeled viral and ^{32}P-labeled cellular marker DNAs (Fig. 12).

The 50 to 90S viral DNA (^3H-labeled) has been found consistently to exhibit a buoyant density in alkaline CsCl gradients intermediate between that of viral and cellular DNA (Fig. 12a). In contrast, the 34S viral DNA isolated from Ad2-infected KB cells by the same technique co-sediments with the viral marker DNA (Fig. 12b). These results can be explained best by the covalent integration of Ad2 DNA into KB cell DNA. This interpretation is strengthened further by experiments demonstrating

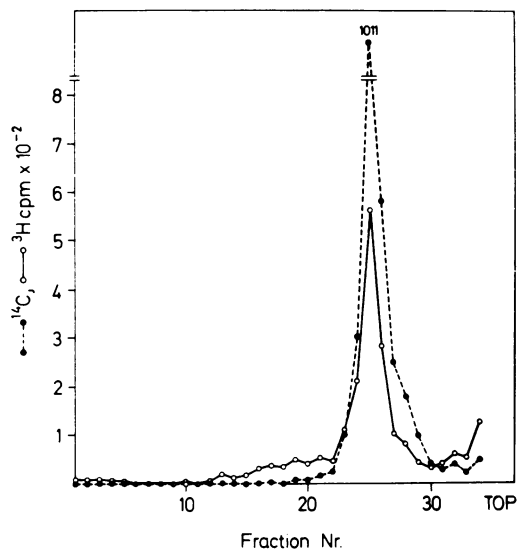


FIG. 8. *Isolation of fast-sedimenting viral DNA from nuclei isolated from Ad2-infected KB cells. KB cells growing in monolayers (1.85 × 10^6 cells) were infected with unlabeled, CsCl-purified Ad2 at an MOI of 100 PFU/cell. The infected cells were labeled with [^3H]thymidine (30 μCi/ml) between 14.5 and 17.5 h postinfection. At the end of the labeling period, the cells were washed with PBS and the nuclei were isolated. Approximately 30,000 nuclei were lysed in alkali on top of an alkaline sucrose density gradient. [^{14}C]Ad2 DNA was added as marker. Lysis proceeded for 24 h at 4 C prior to centrifugation in an SW41 rotor at 35,000 rpm for 140 min at 4 C. After centrifugation, 12-drop fractions were collected from the bottom of the gradient.*

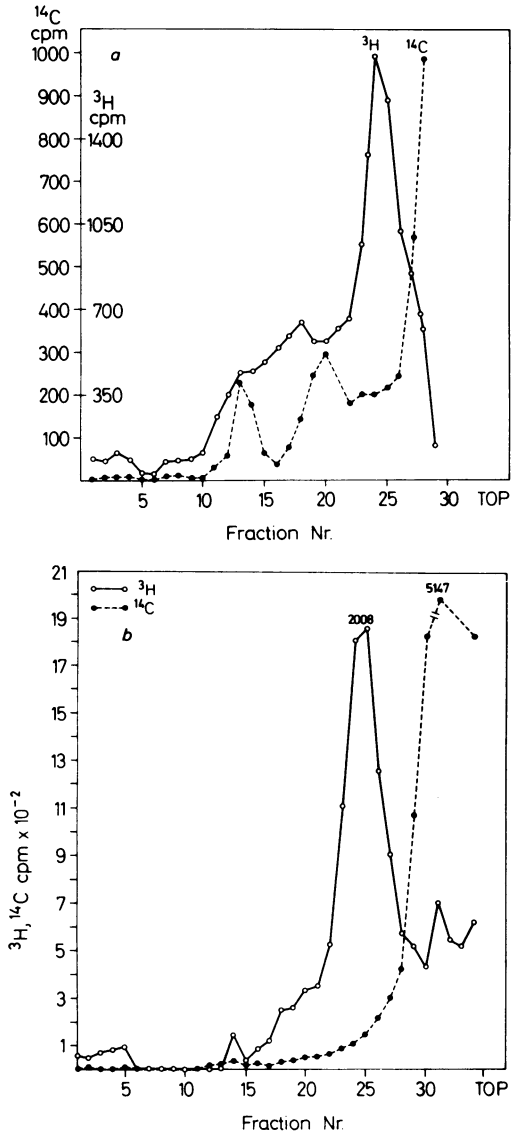


FIG. 9. *Release of protein from fast-sedimenting DNA by extensive alkali treatment. KB cells were infected with unlabeled, CsCl-purified Ad2 at an MOI of 100 PFU/cell. At 13 h postinfection, the Eagle medium containing 10% calf serum was removed and the medium was changed to: 25% Eagle medium containing 10% calf serum, and 75% reinforced Eagle medium without amino acids containing 10% calf serum. In addition, the new medium contained ^{14}C-labeled protein hydrolysate (10 μCi/ml) that was concentrated approximately 10-fold by lyophilization. At 14 h postinfection, [^{3}H]thymidine (30 μCi/ml) was added to the medium. At 17 h postinfection, the cells were washed and lysed in alkali. (a) Alkali lysis at 4 C proceeded for 1 h. The sample was centrifuged in the SW27 rotor at 22,000 rpm for 375 min at 4 C. Fractions were collected and analyzed. (b) Alkali lysis was extended to 17 h at 4 C. The sample was centrifuged in the SW27 rotor at 22,000 rpm for 375 min at 4 C.*

that after ultrasonic treatment of the 50 to 90*S* DNA the ^{3}H-label shifts from the intermediate density position as seen in Fig. 12a to the position of viral DNA and to a lesser extent to that of cellular DNA (Fig. 12c). In a control experiment, viral DNA has been isolated from purified virions and has been incubated at 4 C in alkaline sucrose. Subsequently, the intact viral DNA has been sedimented to equilibrium in an alkaline CsCl density gradient. The viral DNA bands in the viral density stratum (Fig.

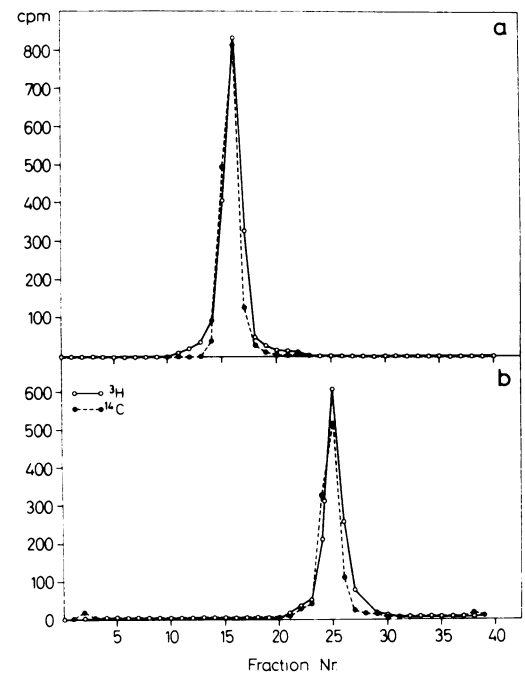


FIG. 10. *Analysis of the fast-sedimenting viral DNA by equilibrium centrifugation in dye-buoyant density gradients. The fast-sedimenting viral DNA was isolated in an experiment similar to the one described in the legend to Fig. 2b. ^{14}C-labeled Ad2 marker DNA was added to the sample, the mixture was adjusted to 0.1 N NaOH and was incubated at 4 C for 15 min. The sample was then neutralized by adding a predetermined amount of 1 N HCl and 1 M Tris-hydrochloride, pH 7.5. The DNA was then analyzed either directly by equilibrium centrifugation in a dye-buoyant density gradient (a) or after dialysis into 0.01 M Tris-hydrochloride, pH 7.2, 0.001 M EDTA, 0.2 M NaCl (b). The samples were centrifuged to equilibrium in the SW56 rotor at 40,000 rpm for 43 h at 20 C. After centrifugation, five-drop fractions were collected and directly analyzed in TM solution. In the figures the densities increase to the left.*

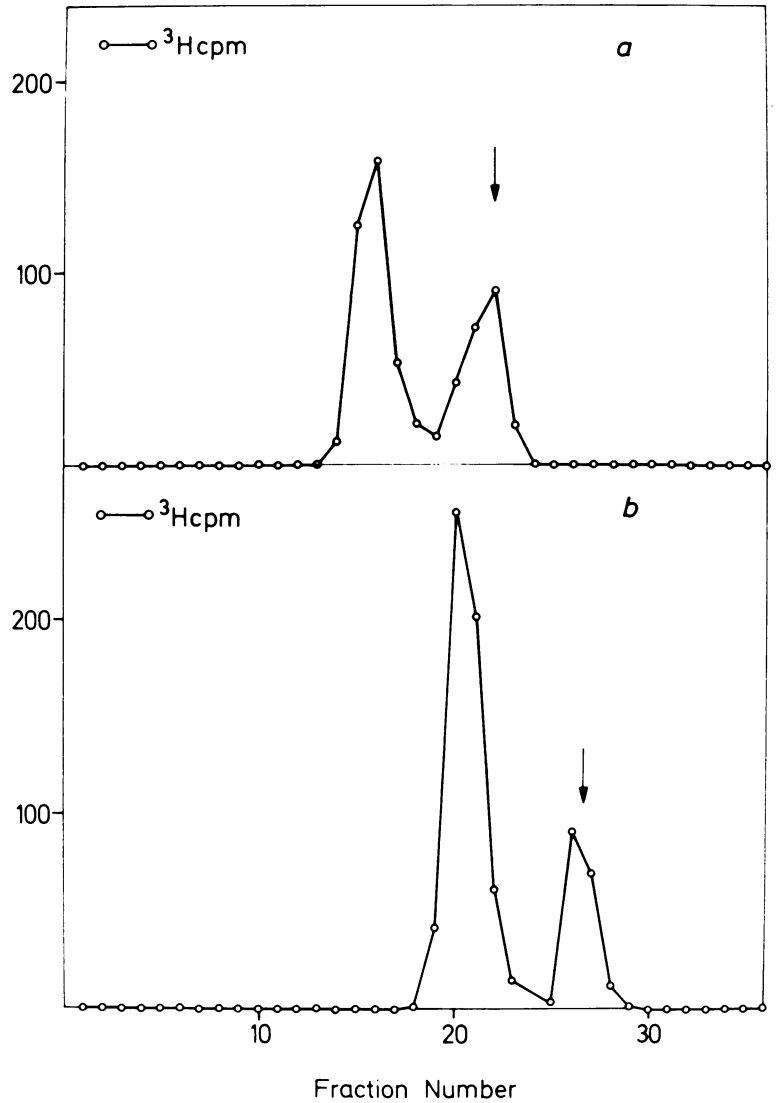


FIG. 11. *Equilibrium sedimentation in dye-buoyant density gradients of SV40 DNA. ^{3}H-labeled SV40 DNA was incubated in (a) 0.5 N NaOH or in (b) 0.01 M Tris-hydrochloride, 0.001 M EDTA, pH 7.2; at 4 C for 19.5 h. Subsequently, sample (a) was neutralized by adding predetermined amounts of 1 M Tris-hydrochloride, pH 7.5, and 1 M HCl. Both samples were then centrifuged to equilibrium in dye-buoyant density gradients by using CsCl propidium iodide as described previously (9). The SW56 rotor of the Spinco L2-65B ultracentrifuge was used: 36,000 rpm for 62 h at 20 C. Four-drop fractions were collected and counted in TM solution as reported elsewhere (10). The arrows indicate the position of ^{14}C-labeled, linear Ad2 marker DNA.*

12d). This finding eliminates the possibility that the density shift observed in alkaline CsCl gradients (Fig. 12a) is due to fragmentation of viral DNA and separation of fragments of different adenine-thymine contents (7). The data presented here demonstrate that the 50 to 90S DNA at least in part represents viral DNA integrated into cellular DNA.

DISCUSSION

This study of the size classes of Ad2 DNA in productively infected cells reports larger amounts of fast-sedimenting, alkali-stable viral DNA than have been seen previously (1, 8). It is likely that only small amounts of the fast-sedimenting DNA have been seen earlier because

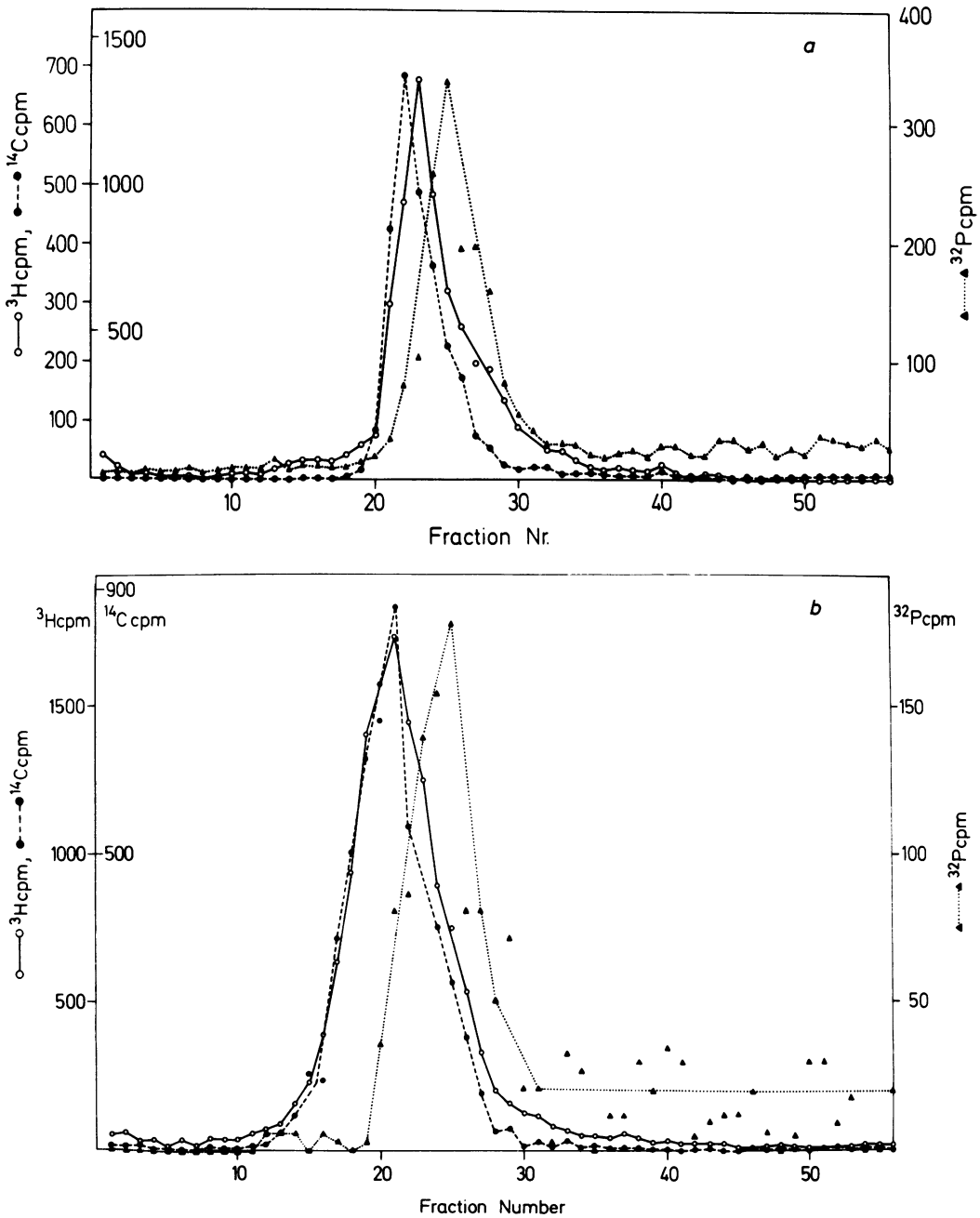
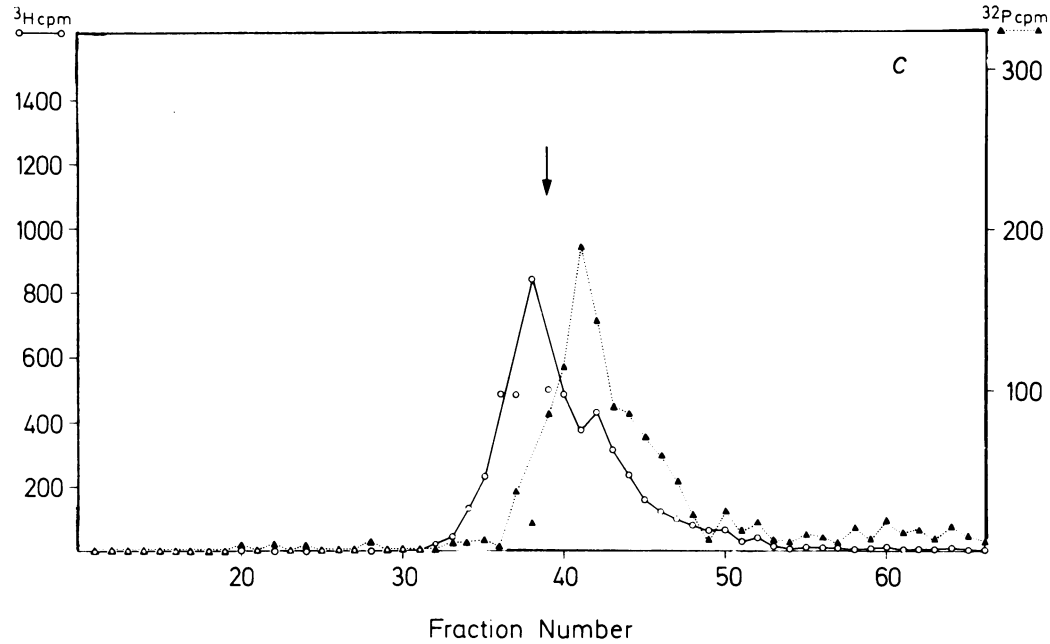


FIG. 12A and B

FIG. 12. *Equilibrium sedimentation in alkaline CsCl density gradients. KB cells were infected with CsCl purified Ad2, labeled with [^{3}H]thymidine (30 μCi/ml), and lysed in alkali, and the intracellular DNA was analyzed in alkaline sucrose density gradients in an experiment similar to the one described in the legend to Fig. 2b. The fractions comprising the 50 to 90S DNA (a) and the 34S DNA (b) were pooled separately and recentrifuged in alkaline CsCl density gradients together with ^{14}C-labeled Ad2 and ^{32}P-labeled KB cell marker DNA under conditions described in Materials and Methods. In another experiment, the 50 to 90S DNA was fragmented by ultrasonic treatment and then was recentrifuged in an alkaline CsCl density gradient with ^{14}C-labeled Ad2 and ^{32}P-labeled KB cell marker DNA (c). The position of the ^{14}C-labeled Ad2 DNA is indicated by an arrow. In a control experiment (d), ^{3}H-labeled Ad2 DNA was extracted from purified virions, incubated in alkaline sucrose for 30 min at 4 C and was then centrifuged to equilibrium in an alkaline CsCl density gradient with ^{14}C-labeled Ad2 marker DNA.*



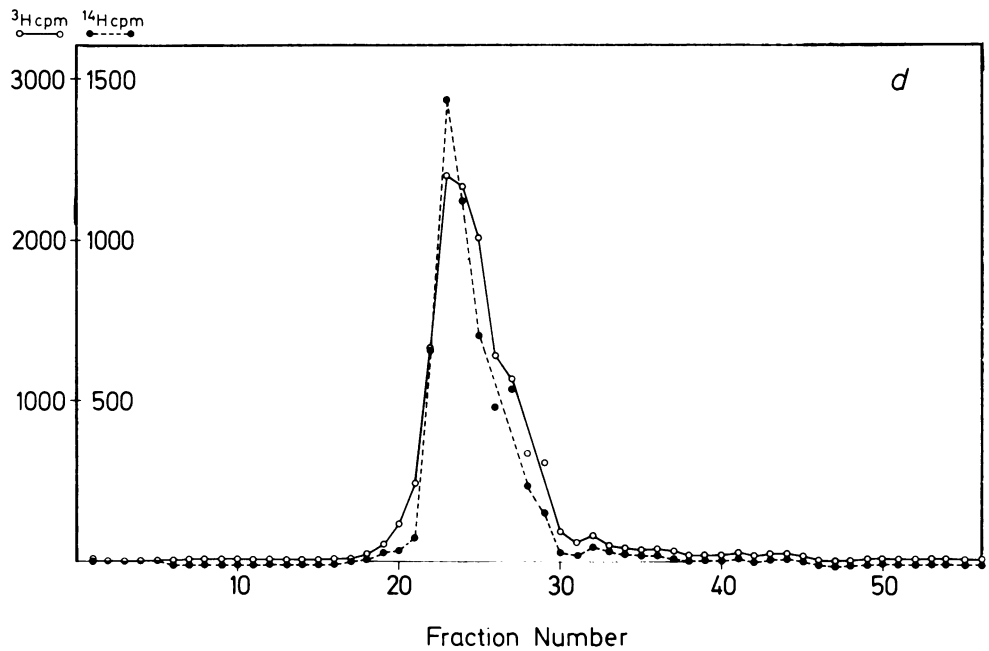


FIG. 12C and D

this DNA has proven to be fragile, and may have been fragmented and lost by the extraction procedure of SDS, Pronase, and phenol used at that time (1). Other studies of adenovirus DNA replication (17, 29, 31; G. D. Pearson, personal communication) do not reveal fast-sedimenting, alkali-stable viral DNA. However, the different methods used in these studies may account for the differences observed. Horwitz (17) also lysed infected cells directly in alkali but used detergents in the gradients and employed different methods for the sedimentation of

DNA. Sussenbach et al. (29) used a nuclear system, and van der Eb (31) removed high molecular weight DNA from the extracts of infected cells prior to sedimentation analysis.

The presence of fast-sedimenting, alkali-stable viral DNA can be explained by one of the following possibilities: (i) mechanical drag of viral DNA into the fast-sedimenting regions caused by the presence of cellular DNA; (ii) association of protein with DNA; (iii) complexing of RNA with DNA; (iv) supercoiled circular viral DNA; (v) linear or circular concatemeric viral DNA; (vi) viral DNA integrated by covalent linkage into cellular DNA.

The present studies show that the last possibility, covalent integration of viral DNA into the cellular genome, is the most likely.

The possibility of mechanical trapping of viral DNA in cellular DNA can be excluded for several reasons. The first is evidence from resedimentation. When fractions containing fast-sedimenting DNA are pooled and recentrifuged on alkaline sucrose gradients, the fast-sedimenting DNA resediments in the same 50 to 90*S* position (Fig. 4). Secondly, viral marker DNA does not show any drag (Fig. 2b). In addition, the use of less than 1 μg of DNA per gradient makes mechanical drag of the DNA unlikely. Furthermore, even when alkaline gradients are overloaded with 200,000 cells per gradient (corresponding to 3.4 μg of DNA) (Fig. 5) or loaded with only 8,000 cells per gradient (corresponding to 0.14 μg of DNA), the relative amounts of 50 to 90*S* DNA are unaltered.

It is not likely that protein remains associated with DNA after extensive alkaline hydrolysis (Fig. 6 and 9). After a short-term incubation of 1 h in alkali, proteins, possibly specific polypeptides, remain bound to the fast-sedimenting DNA. The nature of these polypeptides still has to be determined. However, after a 17-h incubation in alkali, the labeled proteins no longer sediment with the fast-sedimenting DNA. Moreover, after equilibrium sedimentation in alkaline CsCl density gradients at 20 C for 60 h (Fig. 12a), it is highly unlikely that protein could still be bound to the 50 to 90*S* DNA. Other experiments demonstrate that RNA is also digested by 18 h of incubation in 0.5 N alkali at 4 C. Furthermore, the RNA component in the Ad2-specific DNA-RNA complex (10) is digested by incubation in alkali under these conditions. Therefore, complexing of RNA to fast-sedimenting DNA can be eliminated as well.

The results of equilibrium centrifugation of the fast-sedimenting viral DNA in dye-buoyant density gradients demonstrate that this DNA does not consist of supercoiled covalently closed molecules (Fig. 10). It has been shown that SV40 form I DNA is stable during a 20-h incubation in 0.5 N NaOH at 4 C (Fig. 11). Other recently published results employing similar methods (9, 10) demonstrate that covalently closed, supercoiled circular viral DNA cannot be detected in productively or abortively infected cells. Although covalently closed, supercoiled circular viral DNA is not found, it is possible that protein or other intracellular structures maintain the viral DNA in a circular configuration within the cell.

The work of Robinson et al. (27) demonstrates that Ad2 DNA extracted from virions can be maintained in a circular configuration by a protein "linker" which is degraded by Pronase treatment and by a 60-min incubation in alkali (0.2 N NaOH at room temperature). Furthermore, the data of these workers clearly show that after alkali treatment, viral DNA co-sediments with the marker DNA in sucrose density gradients. In the experiments we present here, the 50 to 90*S* viral DNA is treated with 0.5 N NaOH at 4 C for at least 18 h. Thus, it appears very unlikely that a protein linker could still be attached to the 50 to 90*S* viral DNA.

The possibilities of mechanical drag, association of DNA with protein or RNA, and supercoiled circular viral DNA can therefore be ruled out or appear highly unlikely. The fast-sedimenting DNA represents a heterogeneous population of molecules since it sediments in a region extending from 50 to 90*S*. This heterogeneity may result from fragmentation of the molecules and is consistent with the possibilities of both oligomeric and integrated viral DNA.

Results of equilibrium centrifugation of the fast-sedimenting (50 to 90*S*) viral DNA in alkaline CsCl density gradients (Fig. 12a) show that this DNA has a buoyant density intermediate between that of cellular and viral DNA and thus must contain viral and cellular sequences. Upon fragmentation of the DNA by ultrasonic treatment, the ^{3}H-label shifts from the intermediate density position to that of viral DNA and to a lesser extent to that of cellular DNA (Fig. 12c). When viral DNA isolated from purified virions is treated extensively with alkali and then centrifuged to equilibrium in an alkaline CsCl density gradient it cobands with marker DNA. This result makes it unlikely that the hybrid density of the 50 to 90*S* DNA is due to fragmentation and separation of viral DNA fragments with different adenine-thymine content. Unit-size (34*S*) Ad2 DNA isolated from infected cells under identical conditions in alkaline sucrose density gradients cobands in

alkaline CsCl gradients with marker DNA isolated from ^{14}C-labeled virions (Fig. 12b). These findings fail to support the possibility of linear or circular oligomeric viral DNA unlinked to cellular DNA. They do support the model of viral genetic material integrated into host DNA.

The 50 to 90S DNA fails to hybridize within an 18-h period to KB cell DNA fixed to filters (Fig. 3b). It is possible that viral DNA is covalently linked to unique cellular DNA sequences. The data presented in this communication indicate that integration of adenovirus DNA is not restricted to the abortive system (4, 6) but also occurs in productively infected cells. The precise quantitation of the amount of viral DNA requires determinations of the reassociation kinetics with viral and host DNA.

If integration of Ad2 genetic material into cellular DNA does indeed occur as a general phenomenon in productive infection, the biological significance of this event has yet to be determined. Further experiments should explore whether integration may be necessary for viral DNA replication or may be required for efficient late transcription of viral genes using cellular promoter sites. Such promoters may be preferred by the cellular or viral polymerase systems, in both. It must also be determined if integration is a chance event perhaps carrying serious consequences for the cell but no advantage to the virus.

Experimental evidence from work with DNA tumor viruses in the abortive-transformed (2, 4, 6, 15, 28) and productive (19) systems makes it conceivable that integration is a general phenomenon which occurs in productive infection as well. To understand precisely what role integration plays in the abortive and productive systems it is essential to analyze both physically and genetically the site or sites of integration. Finally, it must be determined whether the entire viral genome or only fragments of it become integrated into host cell DNA.

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