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Intracellular forms of adenovirus DNA: Integrated form of adenovirus DNA appears early in productive infection*

(alkaline sucrose gradients/DNA·DNA hybridization/reassociation kinetics/*Eco*RI restriction endonuclease)

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ABSTRACT **In KB cells productively infected with adenovirus type 2, alkali-stable >100S and 40–100S viral DNAs are synthesized starting 2–4 hr postinfection, i.e., before unit length (34 S) viral DNA is made. The amount of >100S and 40–100S viral DNA increases when 34S viral DNA synthesis begins, and at 16–18 hr postinfection, the 40–100S viral DNA represents 5–20% of the total intracellular viral DNA. The 40–100S viral DNA is synthesized throughout infection. Part of the 40–100S DNA synthesized 5–8 hr postinfection has a density in alkaline CsCl gradients intermediate between those of viral and cellular DNAs. This finding indicates that newly synthesized viral DNA is covalently linked to cellular DNA. Viral sequences can be excised from the cellular DNA of infected cells with the *Eco*RI restriction endonuclease. Fragments of viral DNA are detected in polyacrylamide–agarose gels by DNA·DNA hybridization, and these fragments correspond in size to most of the known *Eco*RI fragments of adenovirus 2 DNA. Viral DNA sequences in size-classes between the *Eco*RI-A and -C fragments are also found and probably represent viral DNA linked to cellular sequences.**

In previous publications (1, 2) an alkali-stable, high-molecular-weight (50–90 S) form of viral DNA was described in productively infected cells, and evidence was presented that this DNA represented viral sequences integrated into cellular DNA. Integrated viral sequences in productively infected cells were also described in other virus systems (3). With adenovirus, integrated viral genomes were previously found in abortively infected cells (4–6), and it was shown that in adenovirus-transformed cells specific fragments of the viral genome persisted (7), probably in an integrated form (8).

In this communication, evidence is presented that newly synthesized viral DNA is first detected in the >100S and the 40–100S size-classes between 2 and 4 hr postinfection (p.i.). Part of the 40–100S DNA exhibits a buoyant density in alkaline CsCl gradients intermediate between that of cellular and viral DNA. Viral sequences synthesized 6–12 hr p.i. can be excised from the peak of cellular DNA with the *Eco*RI restriction endonuclease. Some of the fragments generated do not match in size any of the *Eco*RI fragments characteristic of adenovirus 2 DNA. Lastly, it will be shown that after infection of cells with viral DNA, parental viral DNA is converted into 40–100S DNA.

MATERIALS AND METHODS

Cells and Virus. KB (human) cells (9) were grown in monolayer or suspension cultures in Eagle's medium (10) supplemented with 10% calf serum. Adenovirus type 2 (Ad2) was propagated in KB suspension cultures, using published techniques (1, 2, 11, 12).

[^{3}H]Thymidine-Labeled Ad2 of High Specific Radioactivity was obtained by infection of KB cells in suspension culture with Ad2 at a multiplicity of 20 plaque-forming units (PFU) per cell (13) and labeling the culture with 10–15 μCi of [^{3}H]thymidine (25–30 Ci/mmol) per ml.

Analysis of the Intracellular DNA on Alkaline Sucrose Gradients. In some of the experiments, the published procedure (1, 2, 14) was modified in that 10^5 Ad2-infected or mock-infected KB cells per gradient were lysed in 0.5 M NaOH, 0.05 M EDTA for 18 hr at 4° in a plastic tube. Subsequently, the lysate was neutralized by adding a predetermined amount of 5 M HCl, 0.5 M Tris·HCl, and was incubated with 500 μg of autodigested Pronase B per ml at 37° for 30–60 min. The extract was then made 0.5 M in NaOH with 6 M NaOH and was finally layered on top of an alkaline sucrose density gradient. In other experiments the Ad2-infected or mock-infected KB cells were first incubated at 37° with 250 μg of Pronase or proteinase K per ml for 1 or 18 hr at neutral pH, and incubation was subsequently continued in 0.5 M NaOH at 4° for 18 hr (in the case of 1 hr incubation with Pronase) or the lysate was layered on an alkaline sucrose gradient immediately after adding alkali (in the case of 18 hr incubation with Pronase).

Resedimentation of the 40–100S Viral DNA. The 40–100S DNA was prepared by zone velocity sedimentation in alkaline sucrose density gradients and resedimented as described previously (1).

Analysis of Cellular DNA from Ad2-Infected KB Cells by Specific Cleavage with *Eco*RI Restriction Endonuclease and DNA·DNA Hybridization. KB cells growing in monolayer cultures were infected with Ad2 (100 PFU per cell) and labeled with [^{3}H]thymidine (100 μCi/ml) from 6 to 12 hr p.i. At the end of the labeling period, nuclei were prepared (15), the nuclear DNA was extracted by the sodium dodecyl sulfate–Pronase–phenol method (14), and the peak of the cellular DNA was isolated on a neutral sucrose gradient (Fig. 7a). Samples of 0.5–1.0 ml of cellular DNA (1–2 μg) were dialyzed into 0.01 M Tris·HCl, pH 7.5, 0.001 M EDTA. Subsequently, 2–5 μg of ^{14}C-labeled Ad2 marker DNA was added, and the DNA was cleaved with the *Eco*RI restriction endonuclease under conditions outlined elsewhere (2, 12). The digestion products were analyzed by electrophoresis on 13 cm long, cylindrical, 1.5% polyacrylamide–0.8% agarose gels at 40 V for 10–12 hr (12); the gels were cut into 1 mm slices; and the DNA was eluted from each slice by ultrasonic treatment (microtip) for 3 min in 1.0 ml of 0.45 M NaCl, 0.045 M sodium citrate, pH 7.6 (3 × SSC) (16). The ^{3}H- and ^{14}C-labeled DNA from each slice was hybridized to unlabeled Ad2 DNA fixed to nitrocellu-

Abbreviations: p.i., postinfection; Ad2, adenovirus type 2; PFU, plaque-forming units.

* This is part IV of a series. Publication no. III is ref. 1.

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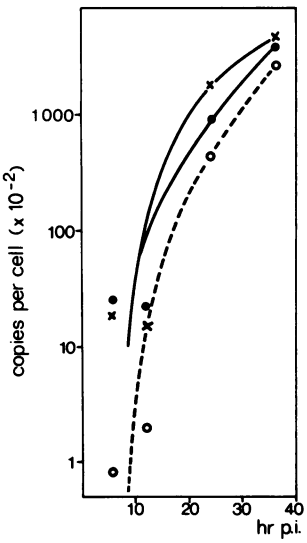

FIG. 1. The amount of viral DNA per Ad2-infected KB cell as a function of cell number per dish and time postinfection. KB cells growing in monolayers were infected in this and all subsequently described experiments with CsCl-purified Ad2 (100 PFU per cell) at cell densities as indicated. At various times p.i., the total intracellular DNA was extracted and the number of Ad2 genome equivalents was determined by measuring the enhancement of reassociation of ^{3}H-labeled Ad2 DNA. Technical details of reassociation kinetics have been described elsewhere (2) and calculations have been performed according to Gelb *et al.* (18). o- - -o, 0.25 × 10^6; ●—●, 0.5 × 10^6; ×—×, 1.0 × 10^6 cells per 60 mm dish at time of inoculation. For most of the experiments reported in this paper, 1 to 2 × 10^6 KB cells in a 60 mm diameter dish were used.

lose filters (17) (Fig. 7c). All hybridization values were corrected for unspecific binding of ^{3}H-labeled DNA to "empty" filters, which was <1% as determined in 10 different fractions across the ^{3}H peak in Fig. 7b.

RESULTS

Quantitation of Intracellular Viral DNA by Reassociation Kinetics. In KB cells infected with Ad2 up to 10^6 viral DNA copies per cell are produced. The amount of viral DNA per cell has been determined at different times after infection by reassociation kinetics (Fig. 1) and, for rapidly growing cells, appears to be independent of the number of cells per plate. At 5.5 hr p.i., up to 2000 viral genome copies per cell were found. This figure reflects the input multiplicity of 100 PFU/cell used, which corresponds to approximately 1000–10,000 particles per cell. At 16–18 hr p.i., more than 80% of the viral DNA is unit size, 34S DNA (Figs. 2e and 5a).

Size-Class Analysis of Intracellular DNA at Various Times after Infection. The size of the newly synthesized DNA in Ad2-infected KB cells was determined at various times p.i. by zone velocity sedimentation in alkaline sucrose density gradients (Fig. 2). The synthesis of 34S viral DNA which cosediments with the virion marker DNA (arrow) is clearly detectable 8–10 hr p.i. (Fig. 4), and is apparent 10–12 hr p.i. in Fig. 2d. At 16–18 hr p.i. (Fig. 2e), the major class of newly synthesized DNA is of viral size, and this pattern is maintained until late in the infection cycle (26–28 hr p.i., not shown). In Ad2-infected KB cells, four size-classes of newly synthesized DNA are observed: (*i*) A usually sharp peak of DNA sedimenting at >100S (e.g., fractions 6 and 7, in Fig. 2b); (*ii*) a heterogeneous class of DNA sedimenting at 40–100 S (e.g., fractions 10–23 in Fig. 2b, or fractions 10–23 in Fig. 2c); (*iii*) a peak of DNA cosedimenting with the ^{14}C marker (Fig 2c, d, and e); and (*iv*) slowly sedimenting material. It is apparent that, even late in infection, newly synthesized DNA is observed which sediments faster than 34S viral DNA. The 40–100S DNA is also present early postinfection (Fig. 2b). The value of 40–100S is an estimate based on the position of the viral marker DNA.

DNA in the 40–60S size-class is also synthesized in mock-infected cells (Fig. 2a). The ^{3}H label in this size-class of DNA can be chased into a peak of >60S cellular DNA (Fig. 3a and b). Hence, the 40–60S DNA represents an intermediate in cellular DNA replication.

At various times p.i., representative fractions from each of the size-classes *i–iii* were pooled and analyzed by DNA·DNA hybridization. The data obtained are indicated by the bars in Fig. 2a–e. The results of many experiments are summarized in Fig. 4 and demonstrate that in >100S and in 40–100S DNA significant amounts of viral DNA can be detected as early as 2–4 hr p.i. Viral DNA sequences of unit length (34 S) cannot be found earlier than 6–8 hr p.i. Thus, >100S and 40–100S viral DNA is detected prior to synthesis of 34S viral DNA and earlier than previously recognized. The amount of >100S and 40–100S DNA increases with the onset of 34S viral DNA synthesis. Even late in infection, large amounts of 40–100S viral DNA are produced.

Resedimentation and Controls. In alkaline sucrose gradients the 40–100S DNA from Ad2-infected KB cells (Fig. 5a) resediments at rates similar to those in the first sedimenta-

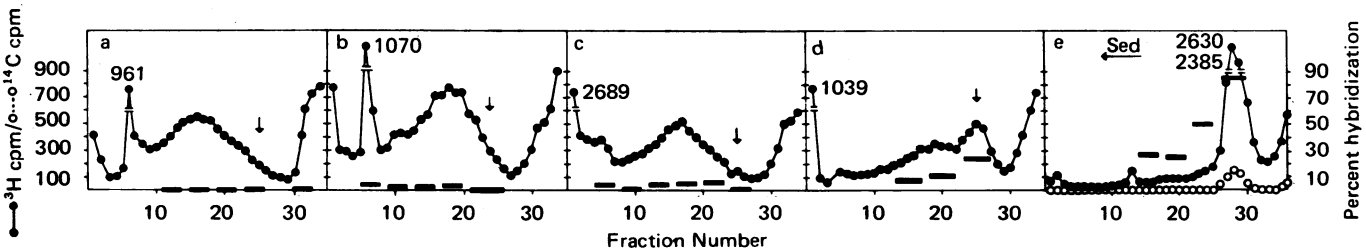

FIG. 2. Size-classes of newly synthesized DNA in mock-infected and Ad2-infected KB cells. KB cells were mock-infected or infected with Ad2 and were labeled with [^{3}H]thymidine (30 μCi/ml) for time intervals indicated below. At the end of the labeling periods, 50,000 cells were lysed in alkali and analyzed by zone sedimentation in alkaline sucrose density gradients. Samples were centrifuged in a Spinco SW41 rotor at 35,000 rpm and 4° for 180 min. In portions of each fraction the ^{3}H and ^{14}C activities were determined. In this and the following figures, the vertical arrows indicate the positions of the ^{14}C-labeled Ad2 marker DNA. The horizontal bars designate the fractions which were pooled, dialyzed, ultrasonically treated, and analyzed for the content of Ad2 DNA by DNA·DNA hybridization. The height of the bars above the abscissa indicates the extent of hybridization of the DNA in corresponding pools: (a) Mock-infection, (b) 2–4 hr p.i., (c) 6–8 hr p.i., (d) 10–12 hr p.i., (e) 16–18 hr p.i. The actual cpm hybridized in each of these experiments were: in (b), 79, 41, 49, 68, 0; in (c), 74, 23, 76, 209, 87, 24; in (d) 320, 223, 867; and in (e) 192, 337, 1015, 11,326. These figures have been corrected for background hybridization and refer to the bars above the abscissa.

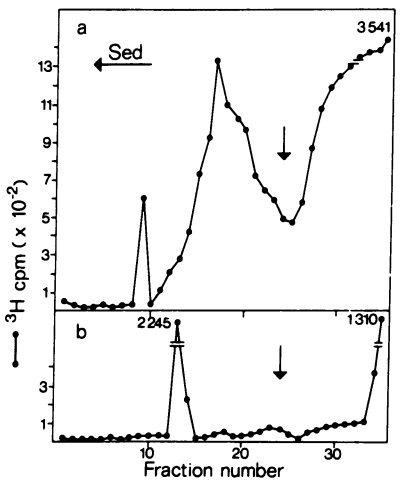

FIG. 3. Pulse-chase experiment with mock-infected KB cells. The DNA in mock-infected KB cells was labeled with [^{3}H]thymidine (60 μCi/ml) from 5–7 hr post mock-infection (p.m.i.). Subsequently, the cells were lysed in alkali and the DNA was analyzed as described (a). In a parallel experiment the cells were washed twice with 37° Eagle's medium–10% calf serum at 7 hr p.m.i., and 5 ml of fresh medium–serum containing unlabeled thymidine (1 μg/ml) were added. The cells were lysed at 16 hr p.m.i. and the DNA was analyzed (b).

tion experiment (Fig. 5b–e), even after dilution and a second resedimentation. The recovery of the radioactively labeled 40–100S DNA after the first resedimentation is 70–100%. Some of the resedimented 40–100S DNA still hybridizes to viral DNA (Fig. 5e).

The occurrence of the 40–100S viral DNA is independent of the lysis procedure used: alkali lysis for 18 hr, alkali lysis

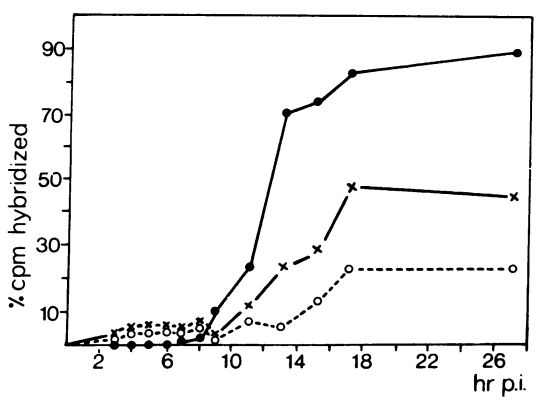

FIG. 4. Analysis by DNA·DNA hybridization of the different size-classes of newly synthesized DNA in Ad2-infected KB cells. KB cells growing in monolayer cultures were infected with Ad2 and were labeled with [^{3}H]thymidine (30 μCi/ml) at time intervals starting 2–4 hr until 26–28 hr p.i., as indicated. At the end of the labeling period, 50,000 cells were lysed in alkali, and the different size-classes of intracellular DNA were separated on alkaline sucrose gradients (see Fig. 2). For each time period, representative fractions were pooled, dialyzed against 0.01 M Tris·HCl, pH 7.2, 0.001 M EDTA, and analyzed for viral sequences by DNA·DNA hybridization (17) to unlabeled Ad2 DNA fixed on filters (3 μg per filter). The value obtained for the 2–4 hr p.i. labeling period is plotted at the 3 hr time point, the one for the 3–5 hr p.i. period at the 4 hr time point, etc. Each value represents the average of 3–7 independent determinations and has been corrected for the background value of 0.11% hybridization of ^{3}H-labeled DNA from mock-infected cells (Fig. 2a). This background value is considered unspecific, since no homology between viral and cellular DNA has been found by reassociation kinetics. O- - -O, >100S DNA; ×—×, 40–100S DNA; ●—●, 34S (viral) DNA.

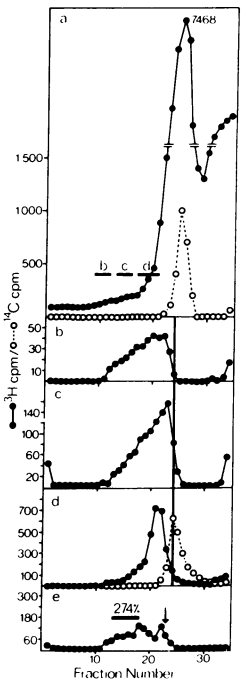

FIG. 5. Resedimentation and hybridization of the 40–100S DNA from Ad2-infected KB cells. (a) The 40–100S DNA from Ad2-infected KB cells which were labeled with [^{3}H]thymidine (100 μCi/ml) between 17 and 19 hr p.i. was prepared as described in the legend to Fig. 2 and resedimented as described. (b–d) The pools in (a) designated b, c, d, and indicated by bars were resedimented as shown in panels (b), (c), and (d), respectively. In the experiments shown in (a) and (d), Ad2 [^{14}C]DNA was used as marker. The vertical lines indicate the positions of the ^{14}C-labeled Ad2 marker DNA in all graphs. (e) The 40–100S DNA from Ad2-infected KB cells which were labeled between 16 and 18 hr p.i. with [^{3}H]thymidine (compare Fig. 5a) was resedimented in alkaline sucrose gradients and the fractions indicated by the bar were pooled and the amount of viral DNA sequences was estimated by the DNA·DNA hybridization procedure (17) as described in the legend to Fig. 2. The results are indicated by the horizontal bar (27.4% of viral DNA). Gradients were centrifuged in the SW41 rotor at 37,000 rpm and 4° for 300 min.

supplemented by digestion of the lysates with Pronase B or proteinase K and subsequent analysis in alkaline gradients, or sodium dodecyl sulfate–Pronase lysis or lysis by proteinase K (19) and subsequent analysis in neutral sucrose gradients yield comparable results (20).

Quantitation of Viral Sequences in the 40–100S DNA by Reassociation Kinetics. The 40–100S DNA from Ad2-infected KB cells was also analyzed by reassociation kinetics (2, 18, 21). So far, the analysis was limited to the 16–18 hr period. The number of viral DNA copies per cell sedimenting in the 40–100S region was found to be between 2000 and 7000 genome equivalents, corresponding to 5–20% of the total intracellular viral DNA at that time (compare Fig. 1). A more extensive investigation of viral sequences in the 40–100S DNA at early times after infection will be published elsewhere (Fanning and Doerfler, in preparation).

Analysis of the 40–100S DNA in Alkaline CsCl Density Gradients. The >100S and 40–100S DNA from Ad2-infected KB cells which were labeled with [^{3}H]thymidine (100 μCi/ml) between 5 and 8 hr p.i. was resedimented to equilibrium in alkaline CsCl density gradients (1, 2, 5). The results are similar to those published earlier (1) in that this size-class of DNA exhibits buoyant densities of viral and cellular DNA, but also contains DNA with buoyant densities in-

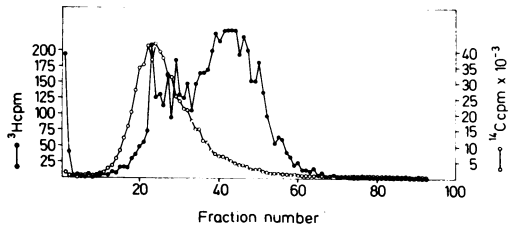

FIG. 6. Equilibrium sedimentation of the >100S DNA in alkaline CsCl density gradients. Ad2-infected KB cells were labeled with 100 μCi of [^{3}H]thymidine between 5 and 8 hr p.i. The >100S DNA was isolated as described in the legend to Fig. 2 and resedimented in alkaline CsCl density gradients as described earlier (1, 2). ^{14}C-Labeled Ad2 DNA was used as density marker. Density increases to the left.

termediate between those of viral and cellular DNAs (Fig. 6). Similar results were found with 40–100S DNA. This finding is consistent with the notion that the >100S and 40–100S DNA contains integrated viral sequences.

Analysis of Cellular DNA from Ad2-Infected KB Cells by Cleavage with *Eco*RI Restriction Endonuclease. Integrated viral sequences were detectable in >100S and 40–100S DNA early after infection. Excision of the viral sequences from cellular DNA with restriction endonucleases and subsequent analysis by gel electrophoresis and DNA·DNA hybridization might yield information about the cellular DNA sequences adjacent to the integrated viral DNA fragments. Ad2-infected KB cells were labeled with [^{3}H]thymidine between 6 and 12 hr p.i., and the cellular DNA was isolated by zone sedimentation in neutral sucrose density gradients (22). The cellular DNA was cleaved with the *Eco*RI restriction endonuclease and the fragments were analyzed (Fig. 7). The sedimentation profile of the intracellular DNA demonstrates that only small amounts of unit size (31 S) viral DNA are present (Fig. 7a). The analysis of the *Eco*RI fragments from the cellular DNA peak (Fig. 7a, fractions 7–17) by agarose–polyacrylamide gel electrophoresis (Fig. 7b) and subsequent DNA·DNA hybridization of the DNA from each gel slice (Fig. 7c) yields the following results: (*1*) Upon cleavage with the *Eco*RI restriction endonuclease, the bulk of the DNA has a size intermediate between that of the *Eco*RI-A (13.6×10^6 daltons) and -B (2.7×10^6 daltons) fragments. DNA larger than the *Eco*RI-A fragment is not found by gel electrophoresis (Fig. 7b) or zone velocity sedimentation. (*2*) Viral DNA corresponding to most of the *Eco*RI fragments is represented in cellular DNA, although in relative proportions different from those in Ad2 virion DNA (Fig. 7c). (*3*) Viral DNA fragments of a size intermediate between that of the *Eco*RI-A and -B, -C and -D, and -E and -F fragments are generated (Fig. 7c). The intermediate size DNA fragments probably contain viral and cellular sequences and originate from the site(s) of integration of viral DNA. The distribution pattern of viral sequences indicates that fragments of viral DNA are integrated rather than the entire viral genome. This interpretation is supported by the finding that the viral sequences from the low-molecular-weight region of the gel, corresponding to *Eco*RI fragments C–F, hybridize to the *Eco*RI-A fragment, whereas the DNA between *Eco*RI fragments A and B contains few sequences homologous to *Eco*RI fragment A (Baczko and Doerfler, in preparation).

Fate of Parental Viral DNA. Since viral DNA synthesized early in infection was found in an integrated form, it was of interest to follow the fate of the parental viral DNA. Upon infection of KB cells with Ad2 DNA (24), a significant

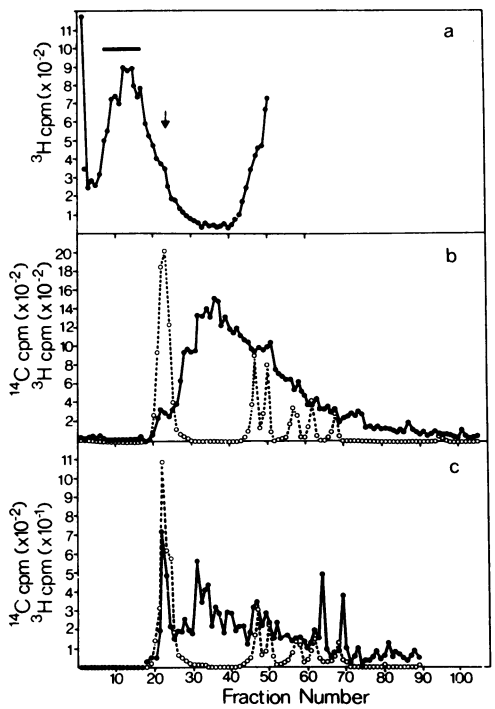

FIG. 7. Analysis by gel electrophoresis of the *Eco*RI restriction enzyme fragments of the cellular DNA peak synthesized 6–12 hr p.i. in Ad2-infected KB cells. For details see *Materials and Methods*. (a) Distribution of the ^{3}H-labeled DNA synthesized 6–12 hr p.i. in a neutral sucrose gradient. Conditions of centrifugation: SW41 rotor, 35,000 rpm. 4°, 360 min. (b) Fractions 7–17 indicated by the horizontal bar (a) were pooled, dialyzed, and concentrated. Subsequently, 2–5 μg of ^{14}C-labeled Ad2 DNA was added and the mixture was incubated with the *Eco*RI restriction endonuclease as described elsewhere (2). The fragmented DNA was analyzed by electrophoresis on polyacrylamide–agarose gels. The labeled DNA from individual gel slices was eluted as described in *Materials and Methods* and 0.1 ml portions were dried on nitrocellulose filters and counted in toluene-based scintillator. ●—●, ^{3}H-labeled DNA (cpm); o- - -o, ^{14}C-labeled Ad2 marker DNA (cpm) representing the six specific *Eco*RI fragments (23). (c) The DNA from each gel slice was hybridized to Ad2 DNA on filters. The ^{3}H and ^{14}C activity curves (symbols as in b) represent the positions of Ad2-specific sequences.

portion of the parental viral DNA was first converted to 19–23S fragments (11) (Fig. 8a and b) and, starting 6 hr p.i., to the 40–100S DNA (Fig. 8b and c). Both the fragmented and the 40–100S forms of DNA can be identified as viral by DNA·DNA hybridization (Fig. 8a–c). The 40–100S parental viral DNA resediments in alkaline gradients in the same size range (Fig. 8d). The data suggest, but do not prove, that the parental viral DNA is first cleaved and subsequently linked covalently to cellular DNA.

DISCUSSION

The data described demonstrate that the >100S and 40–100S viral DNA is synthesized early after the infection. There is evidence from the analysis of DNA from uninfected cells that the 40–100S DNA can serve as an intermediate in DNA synthesis. The 40–100S DNA corresponds to molecules of approximately 32×10^6 to 250×10^6 daltons (25) or of 16–125 μm length. Newly synthesized DNA of 30–180 μm length has been detected, e.g., in mouse L cells (26) and in Chinese hamster ovary cells (27).

The amount of >100S and 40–100S viral DNA increases with the onset of synthesis of unit length (34 S) viral DNA. A

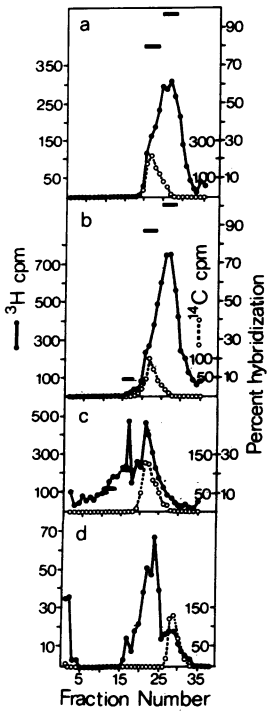

FIG. 8. Fate of the parental Ad2 DNA after infection of KB cells with ^{3}H-labeled Ad2 DNA. (a–c) KB cells growing in monolayers were infected with 2.5 μg of ^{3}H-labeled Ad2 DNA using the Ca^{++}-method (24). At 2 (a), 6 (b), and 24 (c) hr p.i., the intracellular DNA was isolated (14) and sedimented in alkaline sucrose density gradients (1): SW41 rotor, 35,000 rpm, 4°, 300 min. To all gradients ^{14}C-labeled Ad2 marker DNA was added. (d) Resedimentation of fractions 10–18 from an experiment similar to the one described under (c). The alkaline gradient was 15–30% in sucrose; SW41, 38,000 rpm, 4°, 270 min. The horizontal bars designate the fractions which were pooled and hybridized against Ad2 DNA on filters (17). The height of the bars indicates the extent of hybridization.

part of the parental Ad2 DNA is fragmented and subsequently incorporated into 40–100S DNA starting around 6 hr p.i.

The results of an analysis of the 40–100S DNA in alkaline CsCl density gradients and of experiments in which viral DNA fragments were excised from cellular DNA of Ad2-infected cells are consistent with the interpretation that, early after productive infection, viral sequences become covalently linked to cellular DNA. The present data are most compatible with the interpretation that fragments of viral DNA are integrated. The results do not support the model of simple oligomeric forms of viral DNA. Oligomers were observed in the lytic cycle of SV40 (M. A. Martin and P. W. J. Rigby, personal communications). It remains to be determined whether the linkage between viral and host DNA is the result of true integration at specific sites or is rather due to massive recombination between viral and host DNAs. In lytically infected human cells apparently random association of viral DNA with metaphase chromosomes was observed (28). This finding would support our integration model. We cannot rule out the possibility that part of the 40–100S Ad2 DNA can be accounted for by oligomeric or concatenated viral DNA ("replication monsters") or by rearranged viral genomes carrying substitutions with host DNA. Further experiments will be required to determine whether the 40–100S viral DNA has any function in the replication and/or transcription of Ad2 DNA.

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