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Use of Alkaline Sucrose Gradients in a Zonal Rotor to Detect Integrated and Unintegrated Avian Sarcoma Virus-Specific DNA in Cells

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We have attempted to distinguish integrated and unintegrated forms of avian sarcoma virus-specific DNA in cells by sedimentation through an alkaline sucrose gradient in a slowly reorienting zonal rotor. Results obtained with this procedure are similar to those obtained by the more convenient analysis of networks of high-molecular-weight cell DNA. Most, if not all, viral DNA appears completely integrated into the host cell genome in an avian sarcoma virus-transformed mammalian cell and in normal chicken cells (in which viral DNA is genetically transmitted). Fully transformed duck cells and duck embryo fibroblasts infected for 20 to 72 h contain both integrated and unintegrated viral DNA; up to one copy per cell is integrated within 20 h after infection, and four to eight copies are integrated in fully transformed cells. The amount of unintegrated DNA varies but may comprise over 75% of the viral DNA in acutely infected cells and from 20 to 70% of the viral DNA in fully transformed cells. The unintegrated DNA in either case consists principally of duplexes with "minus" strands the length of a subunit of the viral genome (2.5×10^6 to 3×10^6 daltons) and relatively short "plus" strands (0.5×10^6 to 1.0×10^6 daltons).

Several viruses appear capable of inserting their genomes (or DNA copies of their genomes) into the host cell genome by covalent bonds (1, 4-8, 13, 15, 18, 26, 40). In general, the methods available for detecting the integration of small numbers of viral DNA molecules into cell DNA are cumbersome or of dubious rigor. In an attempt to simplify the most rigorous available approach, sedimentation of DNA in alkaline sucrose gradients (26), we have studied the state of avian sarcoma virus (ASV)-specific DNA in cells, using preformed gradients in a slowly reorienting zonal rotor (17, 46). When used in conjunction with sensitive nucleic acid hybridization assays, a single gradient allows definition of sedimentation patterns for integrated and unintegrated DNA present in low numbers of copies per cell. The results are similar to those obtained with a more convenient but less rigorous test for integrated viral DNA, which asks whether viral DNA is linked to networks of high-molecular-weight cell DNA (40).

MATERIALS AND METHODS

Cells and viruses. Peking duck embryo fibroblasts and chicken embryo fibroblasts were grown as described (40); chicken embryos were purchased from Kimber Farms and were not tested for avian tumor virus antigens or for chicken helper factor activity. XC cells, derived from a rat tumor induced by the Prague strain of ASV (31), were obtained from J. Levy (University of California, San Francisco). The B77 strain of ASV was obtained from R. Friis and P. Vogt and was propagated by passage at high multiplicity in chicken embryo cells. When assayed for focus formation on chicken cells, the virus stocks had titers of about 10^7 focus-forming units/ml. However, assays by end point dilution and molecular hybridization demonstrated a 10-fold excess of transformation-defective virus (43) over transforming virus in these stocks (D. Stehelin, D. Fujita, H. E. Varmus, and J. M. Bishop, manuscript in preparation); therefore, the multiplicities of infection (MOI), based upon the focus assays, underestimate the number of infectious units per cell by a factor of about ten. Short-term infections of cells for analysis of viral DNA synthesis were performed at MOI of 1 to 5 focus-forming units/cell at 37 C for 2 to 3 h with 4 μg of polybrene (Aldrich) per ml; virus-containing medium was then replaced by normal medium until the harvest of cells for biochemical analysis. Chronically infected cultures of duck and chicken cells were grown in medium 199, supplemented with 5% calf serum, 1% chicken serum, 10% tryptose phosphate broth, 1% dimethyl sulfoxide, and antibiotics. Titers of ASV produced by such cultures were generally 10^6 to 10^7 focus-forming units/ml per 24 h.

Preparation of cells for centrifugation in a zonal rotor. Cells were labeled with 0.1 to 0.25 μCi of [^{3}H]thymidine (Schwarz/Mann, 12 Ci/mmol) per ml

for at least 24 h prior to collection. After the removal of culture medium, the cells were incubated in Puck saline-EDTA with 0.05% trypsin at 37 C for 5 min, scraped into centrifuge tubes, sedimented at 2,000 rpm for 10 min (in a Sorvall RC3 centrifuge), washed in 0.1 M NaCl-0.02 M Tris (pH 8.0)-0.01 M EDTA (DNA buffer), centrifuged again, and resuspended in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate). Ten milliliters containing 3×10^7 to 4×10^7 cells was generally layered onto the gradient in a Ti-15 zonal rotor.

Conditions of centrifugation. A complete description of the loading, slow reorientation, centrifugation, and unloading procedures for the zonal rotor system has been published (17, 45). Briefly, a 1,660-ml 10 to 30% alkaline sucrose gradient (0.2 M NaOH, 1.8 M NaCl, and 0.01 M Na_2 EDTA) was loaded at rest into a Beckman Ti-15 zonal rotor. On top of this gradient, 40 ml of lysis buffer (1.0 M NaOH, 1.0 M NaCl, and 0.01 M Na_2 EDTA), 20 ml of saline (0.85% NaCl), 10 ml of cell sample, and 80 ml of saline were layered sequentially. The lytic zone, saline, and cell sample were kept at 4 C until loaded. The gradient was slowly reoriented 90° from a vertical to a horizontal position while the rotor speed was advanced to ≈300 rpm (46); the rotor was then accelerated to 2,000 rpm, the air bubble was removed, and the rotor was finally accelerated to a centrifugation speed of 31,500 rpm in a specially modified Beckman L3-50 or L5-50 ultracentrifuge. After centrifugation at 20 C, the rotor was decelerated to 2,000 rpm, and the gradient was collected in 20-ml fractions by displacement with 40% sucrose.

When results from these gradients are plotted as a function of distance sedimented, the geometry of the gradient dictates that 20-ml fractions from regions close to the center of the rotor will represent a greater proportion of the linear gradient than 20-ml fractions from regions near the perimeter (47). All data from the gradients in a zonal rotor are plotted linearly according to distance from the center of the rotor; corresponding fraction numbers are also indicated in every case. Since the shape of the rotor septum produces distorted distances for the first two fractions and these fractions do not contain any detectable DNA, these fractions are not included in the figures.

Processing of fractions from zonal gradients. The position of the [^{3}H]cell DNA and the ^{14}C-labeled single-stranded DNA marker (ca. 165*S*, reference 45) was determined by counting a neutralized 1-ml sample of each 20-ml fraction in 5 ml of PCS (Amersham/Searle). The remainder of each fraction was pooled as indicated for each experiment, neutralized with concentrated HCl (after addition of Tris to 0.1 M), and ethanol precipitated at −20 C by the addition of 50 μg of yeast RNA per ml and 1.5 to 2.0 volumes of ethanol. Precipitates were collected by centrifugation at 16,000 × *g* for at least 15 min, either in an SS34 rotor (in a Sorvall RC2B centrifuge) or in a type 19 rotor (in a Beckman L3-50 ultracentrifuge). The nucleic acids were resuspended in 1 ml of 0.01 M Tris, pH 7.4, and 0.01 M EDTA (TE); 100 μg of calf thymus DNA was added, and the samples were treated at 80 C for 90 to 120 min in 0.3 N NaOH to reduce DNA to fragments of 300 to 400 nucleotides (12, 32), hydrolyze the yeast RNA, and inactivate any contaminating nucleases. After neutralization, fractions were precipitated with ethanol, resuspended in small volumes (20 to 50 μl) of TE, and annealed to labeled 70*S* RNA or complementary DNA (cDNA).

DNA extraction. DNA was extracted from cells by a described procedure (33, 40). In brief, trypsinized cells were lysed with sodium dodecyl sulfate and treated with Pronase; nucleic acids were extracted with phenol and either precipitated with ethanol or dialyzed to remove phenol. After incubation with RNase and Pronase, phenol extraction was repeated and the DNA was again dialyzed against 0.01 M Tris-0.005 M EDTA. Where indicated, cells were first fractionated into nucleus and cytoplasm by published procedures (35) or were fractionated by precipitation of high-molecular-weight DNA with 0.8% sodium dodecyl sulfate-1 M NaCl as described by Hirt (14). For analysis by hybridization, samples were exposed to 0.3 N NaOH for either 20 min at 100 C (32) or 120 min at 80 C (12), neutralized, and ethanol precipitated.

Network tests (40). High-molecular-weight cell DNA (ca. 200 to 500 μg) in 0.01 M Tris-0.005 M EDTA was denatured with heat (100 C, 5 min) or with alkali (0.3 N NaOH, 37 C, 60 min), adjusted to 0.6 M NaCl (at pH 7.4), and incubated for 1 to 2 h at 68 C to a C_0t of 2 to 4 mol/s per liter. Samples were centrifuged for 15 min at either 40,000 rpm in a type 40 rotor or at 30,000 rpm in a type 30 rotor; the supernatant fraction was removed, and the DNA network (a visible pellet) was resuspended in 0.01 M Tris-0.01 M EDTA. Samples were then prepared for hybridization as described above.

Neutral sucrose gradients. DNA samples were layered on 5 to 20% sucrose gradients in 0.01 M NaCl-0.01 M Tris-hydrochloride (pH 7.4)-0.001 M EDTA and centrifuged at 20 C. Fractions were treated with 0.3 N NaOH at 80 C for 2 h, neutralized, ethanol precipitated, and assayed by nucleic acid hybridization.

Nucleic acid hybridization. ASV-specific hybridization reagents included [^{32}P]70*S* RNA (labeled to 10×10^6 to 20×10^6 counts/min per μg with 2 mCi of $^{32}PO_4$ per ml [24]), [^{125}I]70*S* RNA (labeled to 40×10^6 to 50×10^6 counts/min per μg by a modification of the Commerford procedure [35]), or [^{32}P]cDNA (synthesized by detergent-activated DNA polymerase associated with the B77 strain of ASV and labeled to 10^8 counts/min per μg with [^{32}P]dTTP [New England Nuclear Corp.][35]). The 70*S* RNA detects DNA strands of "minus" polarity; the cDNA detects DNA strands of "plus" polarity. As noted above, our stocks of B77 virus used to prepare the hybridization reagents contain a 10-fold excess of transformation-defective virus (43); therefore, the putative transforming sequences of ASV are not detectable with these reagents.

Reaction mixtures contained 500 to 1,500 counts/min of 70*S* RNA or cDNA, 0.6 M NaCl, 0.01 M EDTA, 0.02 M Tris (pH 7.4) per min in 15- to 30-μl volumes sealed in capillaries or covered with mineral oil in plastic tubes. After 60 to 70 h of incuba-

tion at 68 C, annealing was assessed by digestion with single-strand specific nuclease S1 (30, 35) or with 50 μg of pancreatic RNase per ml and 5 U of T1 RNase per ml in 2× SSC (37 C, 1 h). In each experiment, the labeled reagents were incubated under identical conditions with uninfected duck cell DNA (to determine the background for the assay, generally 2 to 10% of the total counts per minute) and with 1 to 20 μg of XC cell DNA. From these controls, we constructed calibration curves to permit the measurement of the amount of viral DNA present in each fraction from the percent annealed. (XC cells contain 20 copies of viral DNA per diploid cell [39, 40] or 0.03 ng of viral DNA per μg; annealing is detectable [ca. 5% above background] with as little as 0.3 μg of XC cell DNA [0.01 ng of viral DNA] and increases in an approximately linear fashion to 20 to 40% with 10 to 20 μg of XC cell DNA [35, 37], depending upon the quality of the hybridization reagent; a typical curve with [^{32}P]70*S* RNA is shown in Fig. 8A.) When [^{32}P]cDNA and [^{125}I]70*S* of high specific activity were available, it was possible to employ both in a single annealing reaction with cell DNA without a significant amount of annealing of the reagents to each other (<5%). In this case, annealing of [^{125}I]70*S* RNA and [^{32}P]cDNA could be assessed simultaneously by digestion with S1 nuclease. Scintillation counting of acid-precipitated nucleic acids was performed in Liquifluor-toluene in a Beckman liquid scintillation, three-channel spectrometer, using variable isosets for the measurement of ^{3}H, ^{14}C, and ^{32}P. ^{125}I-labeled reagents were counted in a Packard gamma counter.

RESULTS

Sedimentation characteristics of unintegrated viral DNA. Our colleagues and we have recently described the principal forms of viral DNA synthesized upon infection of duck cells with ASV: linear (or open circular) duplex DNA with genome length "minus" strands (ca. 2.5 × 10^6 to 3 × 10^6 daltons) and segmented "plus" strands (ca. 0.5 × 10^6 to 1 × 10^6 daltons) and supercoiled, closed circular duplexes of about 6 × 10^6 daltons (12, 34, 34a; R. V. Guntaka, O. C. Richards, P. R. Shank, J. M. Bishop, and H. E. Varmus, manuscript in preparation). DNA with similar characteristics is found in cells infected with murine leukemia virus (9, 10). We have confirmed the existence of these forms of ASV-infected duck cells by analysis in an alkaline sucrose gradient in a Ti-15 zonal rotor (Fig. 1). Cells were infected for 8 h and lysed during loading onto the gradient; fractions from the gradient were then assessed for strands of viral DNA by nucleic acid hybridization. The experiment minimized the dangers of artifacts that might arise during the extraction of DNA and provided a reasonable test of our ability to recover and measure unintegrated forms of viral DNA from the large fractions from these gradients. In the gradient shown in Fig. 1, resolution of species of unintegrated viral DNA was maximized by prolonged sedimentation; as a result, over 80% of high-molecular-weight cell DNA (labeled with [^{14}C]thymidine) was pelleted against the sides of the rotor, and no attempt was made to assess integration. About 20% of the unintegrated viral DNA sedimented at about 60–65*S*, as estimated from the sedimentation of simian virus 40 (SV40) (form I) DNA centrifuged under identical conditions in another gradient. The rapidly sedimenting ASV DNA contained equal amounts of "plus" and "minus" strands, and we assume it represents the supercoiled circular form that we have documented elsewhere by

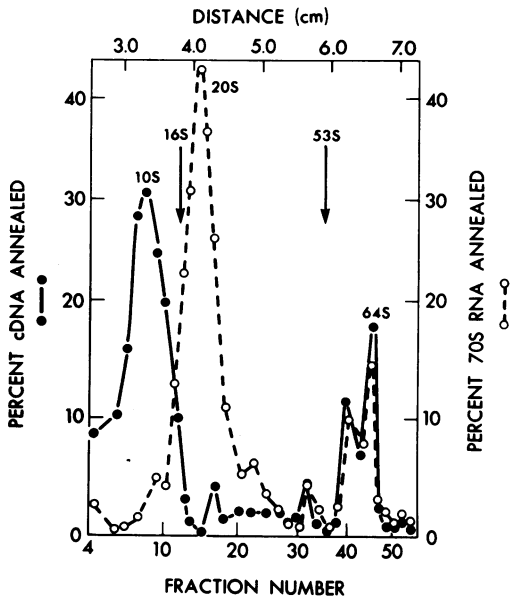

FIG. 1. *Analysis of unintegrated viral DNA from acutely infected duck cells in alkaline sucrose gradients in the zonal rotor. Duck embryo fibroblasts (tertiary cultures labeled with [^{14}C]thymidine) were infected for 8 h with B77 ASV (MOI = 3) and 10^8 cells were lysed and sedimented in a 10 to 30% alkaline sucrose gradient for 12.5 h at 27,000 rpm in a Ti-15 zonal rotor. Under these conditions, over 80% of the [^{14}C]thymidine-labeled cell DNA was recovered against the wall of the rotor. Single fractions (3–16) or pools of two fractions (17–18 to 55–56) from the linear region of the gradient were hybridized with a mixture of ASV [^{32}P]cDNA(●) and [^{125}I]70S RNA (○), as described in the text. The percentage of each reagent annealed is plotted according to the distance sedimented in the gradient. The arrows indicate the positions of [^{3}H]thymidine-labeled SV40 DNA (forms II[16S] and I [53S]) centrifuged under identical conditions in a separate gradient.*

both buoyant density centrifugation and sedimentation in alkaline sucrose gradients in conventional rotors (12). The majority of the viral DNA sedimented more slowly, the "minus" strands in a position (ca. 20S) expected for a full-length copy of the viral genome (ca. 2.5 × 10^6 to 3.0 × 10^6 daltons) and the "plus" strands migrating as relatively low-molecular-weight DNA of about 0.5 × 10^6 to 1.0 × 10^6 daltons. These findings support our contention that an unusual duplex molecule with full-length "minus" strands and segmented "plus" strands of viral DNA is synthesized by viral polymerase and then closed to form a supercoiled molecule that may be the integrating species (12, 34a). This experiment does not, of course, establish a precursor-product relationship between the slowly sedimenting and supercoiled forms.

Sedimentation characteristics of cell DNA. To study the integration of viral DNA in alkaline sucrose gradients, it is necessary to separate peaks of nonintegrated viral DNA and cell DNA, using as much cell DNA as possible to facilitate the detection of viral sequences, yet avoiding artifacts due to overloading the gradients. In preliminary experiments with [^{3}H]thymidine-labeled normal duck fibroblasts, we observed that up to 4 × 10^7 cells could be safely analyzed in a single 1,660-ml alkaline sucrose gradient in a zonal Ti-15 rotor. With larger numbers of cells, gels of DNA were occasionally formed, causing readily apparent distortions of the sedimentation profiles. In some cases, fast-sedimenting aggregates of DNA were found as peaks ahead of the main band of DNA or as pellets against the wall of the rotor. In other cases, particularly with more severe overloading, the gels sedimented extremely slowly and could be observed visually during collection. Elimination of these problems in slow reorienting zonal rotor investigations has been previously discussed (17, 45–47). The bulk of cell DNA of avian origin sediments in these gradients with a DNA marker (produced by irradiating Chinese hamster ovary cells with 1 krad of X ray) whose peak sedimentation coefficient has been established as approximately 165S (45) (Fig. 2–6). The molecular weight of such strands is estimated to be about 5 × 10^8 (19, 20, 45). In all cases, we observed a variable amount of cell DNA (5 to 25%) that sedimented very slowly in these gradients. This DNA may, in part, represent replication units from these growing cells, DNA broken by hydrodynamic shear during the loading steps and lysis, and/or DNA trapped with other cell components.

To test whether cell DNA of very high molecular weight was capable of trapping linear or

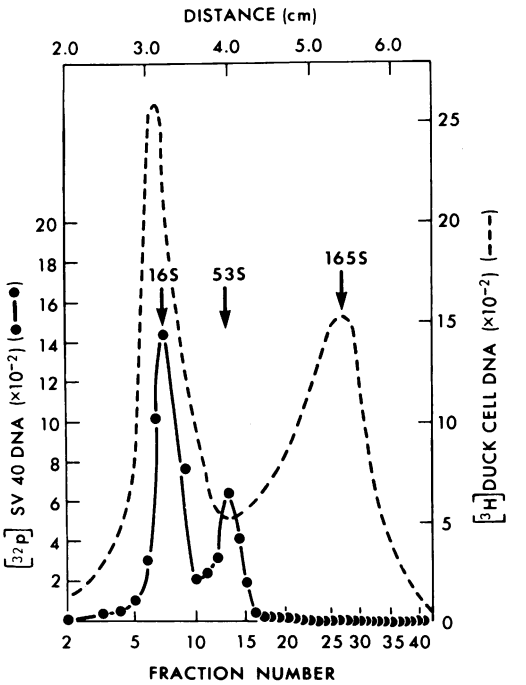

FIG. 2. *Test for trapping of viral DNA by high-molecular-weight cell DNA in alkaline sucrose gradients in the zonal rotor. ^{32}P-labeled SV40 DNA (●) (25 × 10^3 counts of forms I and II per min, kindly provided by N. Mantei and H. Goodman) was mixed with 3.1 × 10^7 duck embryo fibroblasts (-----) labeled with 0.25 μCi of [^{3}H]thymidine per ml in 10 ml of 1× SSC and loaded onto a 10 to 30% alkaline sucrose gradient in a Ti-15 zonal rotor as described in the text. After sedimentation for 3.25 h at 31,500 rpm in a Beckman L3-50 ultracentrifuge, 20-ml fractions were collected, acid precipitated, and counted in a liquid scintillation counter.*

circular forms of DNA of relatively low molecular weight, 4 × 10^7 duck fibroblasts (labeled with [^{3}H]thymidine) and ^{32}P-labeled SV40 DNA (forms I and II) were sedimented in an alkaline sucrose gradient in the zonal rotor (Fig. 2). No detectable [^{32}P]viral DNA (less than 1% of the total) was found in the region of high-molecular-weight cell DNA; we also observed excellent resolution of the supercoiled viral DNA (53S) from the single-stranded circular and linear forms (14–16S). These results indicate that low-molecular-weight DNA does not form aggregates with high-molecular-weight strands under these sedimentation conditions and that the gradients should afford adequate resolution of integrated forms from the described unintegrated forms of ASV DNA.

Integrated ASV DNA in mammalian cells. We have previously determined that the XC cell, derived from a rat tumor induced by the

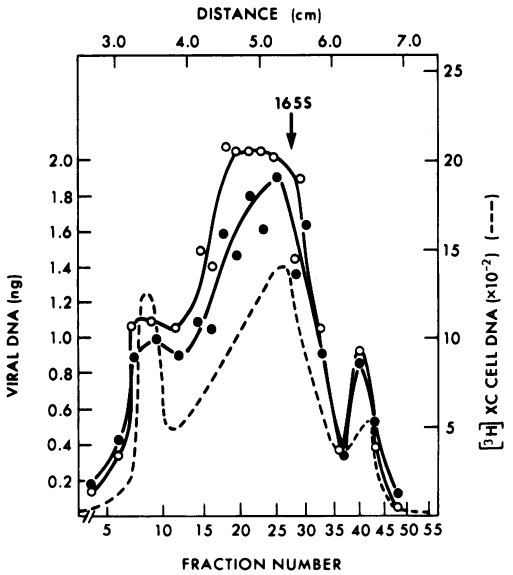

FIG. 3. *Demonstration of integrated ASV DNA in XC cells. Cells (3 × 10⁷), labeled with 0.25 μCi of [³H]thymidine per ml for 48 h, were sedimented for 3.25 h at 31,500 rpm in a 10 to 30% alkaline sucrose gradient in a Ti-15 zonal rotor. Samples (1 ml) from each fraction were counted to determine the profile of [³H]cell DNA (-----) and the position of a ¹⁴C-labeled 165S DNA marker (arrow). DNA was recovered from pools of two fractions (3-4 to 29-30) or four fractions (31-34 to 51-54); half was annealed to [³²P]70S RNA (○) and half was annealed to [³²P]cDNA (●). The amount of viral DNA (in nanograms) was computed from parallel annealings with various amounts of unfractionated XC cell DNA.*

Prague strain of ASV (31), contains approximately 20 copies of ASV DNA per diploid cell (39, 40); all of the viral DNA in this cell appears to be covalently linked to the cell genome, as judged from the inclusion of viral DNA in networks of XC cell DNA (40). The relatively large number of presumably integrated copies of viral DNA made this an attractive cell with which to test the utility of the zonal rotor for analysis of integrated viral genomes. The rat cell DNA, like avian DNA, banded broadly at about 165*S*, with a small amount of DNA near the origin of the gradient (Fig. 3). Hybridization of DNA recovered from the gradient with [³²P]70*S* RNA and with [³²P]cDNA indicated cosedimentation of both "plus" and "minus" strands of viral DNA with cell DNA throughout the gradient. Of course, since perhaps one-third of both viral and cellular DNA migrated with a sedimentation coefficient of less than 70*S*, we cannot exclude the possibility that a minor portion of the viral DNA in these cells is in the form of linear or closed circular, unit length molecules. However, viral DNA sedimenting faster than 70-80*S* must either be integrated into the host cell genome by alkali-stable bonds or represent multimeric closed circles of viral DNA; we consider the latter possibility unlikely. Linear tandem repeats of 20 copies of ASV DNA would form single strands of 60 × 10⁶ daltons and sediment at no faster than about 68*S*. We conclude that this experiment supports our previous contention that most, if not all, ASV-specific DNA in nonpermissive (mammalian) cells is covalently integrated into host cell DNA.

Integrated endogenous viral DNA in normal chicken cells. A number of laboratories have detected DNA homologous to the genomes of avian RNA tumor viruses in uninfected chicken cells (3, 16, 22, 25, 27, 37, 42); most estimates indicate that about one to five copies are present per diploid genome, with the viral DNA coding for an endogenous virus called RAV-0 and perhaps for other viral genes (44). Baluda and co-workers have shown these endogenous, genetically transmitted viral se-

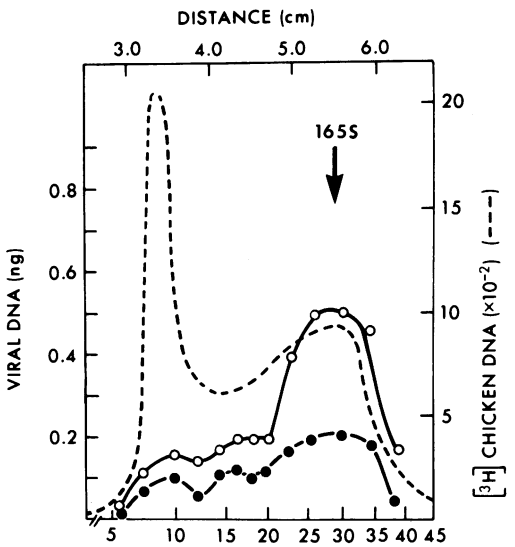

FIG. 4. *Demonstration of integrated viral DNA in normal chicken embryo cells in an alkaline sucrose gradient in the zonal rotor. Normal chicken embryo fibroblasts from secondary cultures (4 × 10⁷ cells, labeled with 0.25 μCi of [³H]thymidine per ml) were sedimented for 3.25 h at 31,500 rpm in a 10 to 30% alkaline sucrose gradient in the zonal rotor. DNA recovered from pools of two fractions (3-4 to 19-20) or four fractions 21-25 to 37-40) was annealed with [³²P]70S RNA (○) or [³²P]cDNA (●) to measure the amounts of "minus" and "plus" strands of viral DNA, respectively. The profile of [³H]cell DNA (-----) is also displayed.*

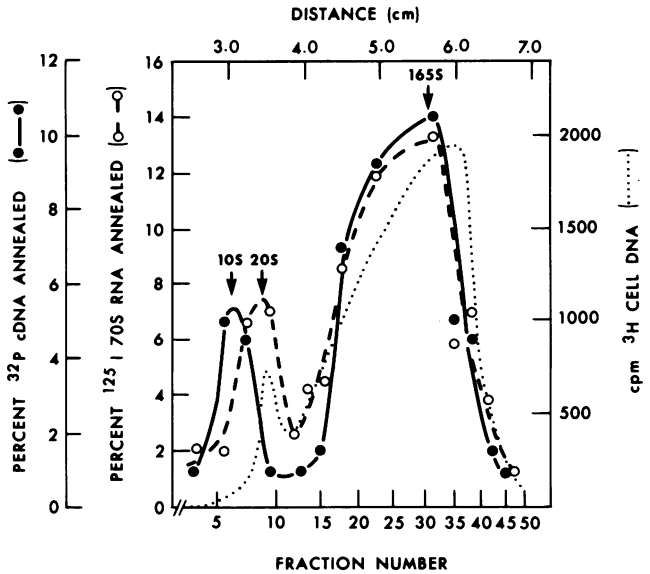

FIG. 5. *Detection of integrated and unintegrated ASV DNA in duck cells transformed by B77 ASV. Duck embryo fibroblasts infected by B77 ASV were passaged four times over the course of 3 weeks; after labeling for 48 h with 0.25 μCi of [³H]thymidine per ml, 3 × 10⁷ cells were layered onto a 10 to 30% alkaline sucrose gradient in the zonal rotor and sedimented for 3.25 h at 31,500 rpm. Samples of fractions were counted to determine the sedimentation of cell DNA (-----), and then DNA was recovered from pools of two fractions (3-4 to 15-16) or four fractions (17-20 to 45-48) and annealed with [¹²⁵I]70S RNA (○) and [³²P]cDNA (●).*

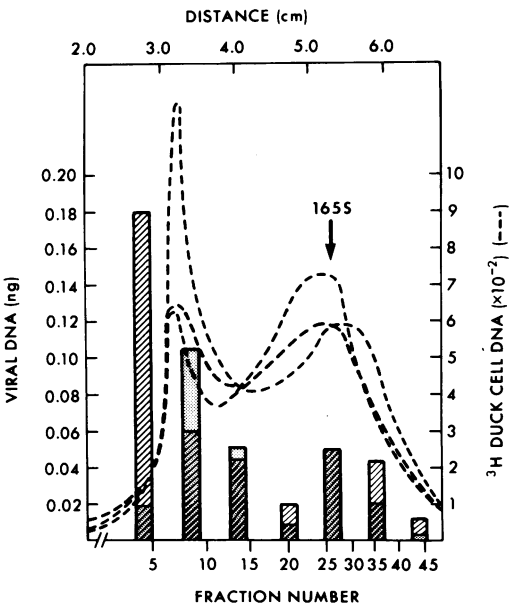

FIG. 6. *Detection of integrated and unintegrated ASV DNA in duck cells acutely infected by ASV. Secondary cultures of duck embryo fibroblasts (labeled with 0.25 μCi of [³H]thymidine per ml) were infected when approaching confluence (ca. 4 × 10⁶ cells/100-mm dish) with B77 ASV in the presence of 4 μg of polybrene per ml (MOI = 3 focus-forming units/cell). After 2 h, the infecting medium was removed and replaced with fresh culture medium. Eighteen hours later, 4 × 10⁷ cells were loaded onto a 10 to 30% alkaline sucrose gradient in the zonal rotor and sedimented for 3.25 h at 31,500 rpm in a Beckman L3-50 ultracentrifuge. Three such gradients were run under similar conditions, and the profiles of [³H]cell DNA were determined by counting aliquots from each fraction (-----). Seven pools were then made from the three gradients (fractions 3 to 6, 7 to 10, 11 to 16, 17 to 22, 23 to 30, 31 to 38, and 39 to 46). After neutralization and ethanol precipitation, the pools were digested with Pronase (500 μg/ml for 60 min at 37 C), extracted with phenol, ethanol precipitated, treated with alkali (0.3 N NaOH, 80 C, 120 min), neutralized, and reprecipitated with ethanol. Portions of each pool were then annealed to [³²P]70S RNA and (in duplicate) to [³²P]cDNA. Amounts of "minus" strands (stippled area of bars) and "plus" strands (striped area of bars) detected per gradient (4 × 10⁷ cells) are illustrated for each region of the gradients.*

quences to be integrated by sedimentation of DNA in alkaline sucrose gradients in conventional rotors and by analysis of buoyant density in cesium chloride gradients (21, 28). We have confirmed their observations with a single gradient in the zonal rotor, using cells from uninfected chicken embryo fibroblasts (Fig. 4). At least two-thirds of viral DNA, detected with either 70S RNA or cDNA (both prepared from B77 ASV), sedimented with high-molecular-

weight chicken cell DNA. The significance of the apparently greater amount of "minus" strand than "plus" strand in this gradient is unclear, and the discrepancy has not been further investigated. The difference may, however, be artifactual, since we calibrate the assays with XC DNA, into which ASV DNA has been introduced by infection. The rate and extent of reaction of our hybridization reagents to viral sequences endogenous to normal chicken cells may differ from the annealing to ASV sequences in XC DNA; since the distribution of viral sequences in 70S RNA and cDNA differs, the measurement of the two strands could be unequally affected.

Integrated and unintegrated ASV DNA in transformed duck cells. We have made extensive use of duck embryo fibroblasts as a host cell for ASV because nucleic acids from the uninfected cells do not show evidence of homology with ASV-specific reagents in conventional tests (37, 40, 41). (Duck cells do, however, contain endogenous sequences related to the putative transforming gene[s] of ASV [29a], but these sequences are not detectable in the annealings performed in this report since they are under-represented in the hybridization reagents [see above]). Fully transformed, virus-producing cultures of infected duck cells have at least four to six copies of viral DNA that we have previously found to be included in networks prepared with the DNA from such cells (40, 41).

To determine whether the linkage we previously observed was alkali-stable and to ask whether any viral DNA remained in an unintegrated form, we subjected DNA from ASV-transformed duck cells to analysis in alkaline sucrose gradients in the zonal rotor (Fig. 5). As expected, strands of DNA containing viral sequences of both "plus" and "minus" polarity were found to sediment very similarly to cell DNA, with a peak at about 165S, indicating that covalently integrated, alkali-stable viral DNA was present in these cells. In addition, however, we observed a significant amount of viral DNA (about 20% in the gradient shown in Fig. 5) in positions expected for unintegrated viral DNA. Moreover, the slowly sedimenting viral DNA consisted of short "plus" strands (6-10S) and longer "minus" strands (ca. 20S); these molecules resemble those seen early after infection, and we take them to be a "signature" of DNA synthesis from an RNA template by viral DNA polymerase. (Fragmented pieces of integrated viral DNA would be expected to contain "plus" and "minus" strands of equal size.) Since the cells employed in this experiment have divided at least 10 times since the initial infection, it is extremely unlikely that the unintegrated forms represent residual DNA synthesized shortly after infection but never integrated. In an accompanying manuscript, we demonstrate that the unintegrated DNA found in these cells is newly synthesized by viral reverse transcriptase in the cytoplasm of transformed duck cells (38).

Integrated and unintegrated ASV DNA in acutely infected duck cells. We have demonstrated elsewhere that viral DNA is synthesized in the cytoplasm of duck cells for at least 6 h after infection with ASV, that viral DNA is subsequently found in the nucleus, and that integration (as measured by the network test) can be detected as early as 9 h after infection (34, 35, 40, 41). Within the first 24 h after infection, we have observed 0.1 to 1 copy of viral DNA integrated per cell, with an increase to 4 to 6 integrated copies after several days (12, 40, 41). Although we have described experiments in which all the viral DNA in the cell 24 h after infection appeared to be integrated, we have also observed, in subsequent experiments designed to study the mechanism of integration, appreciable variation in the percent integrated. By several criteria—Hirt fractionation, network test, cell fractionation, and density sedimentation of bromodeoxyuridine-labeled DNA in CsCl—we have established that as much as 75% of viral DNA may remain in both the cytoplasm and nucleus in an unintegrated state 20 to 24 h after infection (e.g., Fig. 7 and Fig. 8C and D; also unpublished data of H. E. Varmus and S. Heasley). Unintegrated DNA has also been reported in chicken cells as long as 96 h after infection by ASV or avian myeloblastosis virus (2).

We have observed both integrated and unintegrated viral DNA in cells infected for 20 h by sedimentation of DNA in alkaline sucrose gradients in the zonal rotor (Fig. 6). Similar results were obtained with cells infected for 48 or 72 h (unpublished data). Because the number of copies of integrated viral genomes is low in these cells (less than one copy per cell), it was ultimately necessary to pool multiple fractions from three gradients of cells infected for 20 h to obtain adequate annealing in duplicate samples and to study the sedimentation of both strands (Fig. 6). When the amount of viral DNA in the seven pools was analyzed, 30 to 35% of both strands was found to sediment with high-molecular-weight cell DNA (sedimentation coefficient of greater than 100S). We assume this material to be covalently integrated into host cell DNA. The remainder sediments

slowly, with the "plus" strands principally in the 0–20S and the "minus" strands mainly in the 20–60S pools from the gradients.

We have determined the size of the strands of unintegrated DNA in duck cells infected for 22 h by sedimentation of denatured DNA from a Hirt supernatant fraction in a neutral sucrose gradient in a conventional rotor (Fig. 7). Under these conditions, the "minus" strands band broadly at about 27S and the "plus" strands sediment at about 8–10S; the S values are consistent with molecular weights of about 3×10^6 and 0.5×10^6 to 1.0×10^6 for these strands. The absence of rapidly sedimenting "plus" strand DNA in this experiment suggests that little, if any, closed circular viral DNA is present in an unintegrated form at this point after infection. (In duck cells, we have found supercoiled DNA to be most abundant between 7 and 10 h after infection, whereas in a certain line of quail tumor cells, several copies of unintegrated supercoiled viral DNA are found to persist in the nucleus for weeks after the initial infection (R. V. Guntaka, O. Richards, R. P. Shank, J. M. Bishop, and H. E. Varmus, manuscripts in preparation).

Detection of unintegrated DNA in infected duck cells with the network procedure. In earlier communications we have employed the network test to demonstrate integration of ASV DNA in fully transformed and acutely infected duck cells (12, 16, 40, 41). Since we did not observe unintegrated viral DNA in transformed cells in those experiments (40, 41) and, in some cases, reported complete integration of viral DNA within 24 h after infection (12), we asked whether the network test was capable of detecting the unintegrated DNA we observed in alkaline sucrose gradients in the zonal rotor. In addition, at the suggestion of Bellett (4), we prepared networks from alkali-denatured, as well as thermally denatured, DNA to exclude the possibility that the network test, as normally performed, was scoring viral DNA linked to cell DNA by alkali-labile bonds. (A minimum estimate for the rate of hydrolysis under these conditions suggests that the majority of oligoribonucleotides longer than five nucleotides would be cleaved [11].) In contrast to earlier experiments, in which viral DNA was measured by its ability to accelerate the reassociation of labeled duplex DNA synthesized in vitro by viral polymerase, the assays reported here were performed with the reagents used to analyze gradient fractions, labeled cDNA and 70S RNA. As in analysis of gradient fractions, the amount of viral DNA was determined from a calibration curve with XC cell DNA (see above).

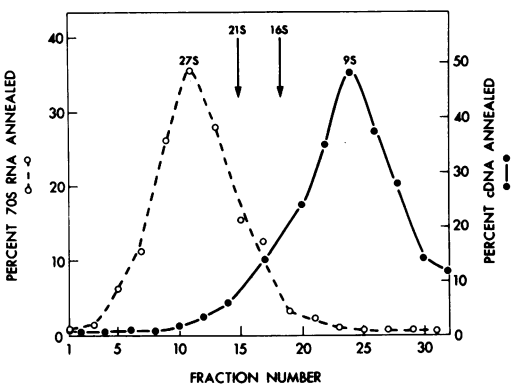

FIG. 7. *Size of strands of unintegrated DNA in acutely infected duck cells. Secondary cultures of duck embryo fibroblast were infected (MOI = 3) for 22 h with B77 ASV. A sodium dodecyl sulfate-salt fractionation was carried out according to a modification of the Hirt procedure (12, 14). DNA was extracted from the supernatant fraction, denatured (100 C, 3 min), and sedimented in a 5 to 20% neutral sucrose gradient for 6.5 h at 35,000 rpm and 20 C in a Spinco SW41 rotor. After treatment with alkali (0.3 M NaOH, 80 C, 120 min), alternate fractions were annealed with [^{32}P]cDNA (●) or [^{125}I]70S RNA (○) to detect "plus" or "minus" strands of ASV DNA, respectively. The positions of forms of [^{32}P]SV40 DNA (form I, 21S; form II, 16S) in a parallel gradient are indicated by arrows. The direction of sedimentation is from right to left.*

DNA from XC cells, fully transformed duck cells, and duck cells infected for 24 h with ASV was used to form networks after denaturation by alkali treatment (0.3 N NaOH, 37 C, 1 h) or by heat (100 C, 5 min). In the case of the acutely infected cells, networks were prepared from both whole cells and nuclei. Increasing amounts of DNA from network and supernatant fractions (and, in some cases, unfractionated cell DNA) were tested for their ability to anneal labeled 70S RNA (Fig. 8); in all cases, similar results were observed with cDNA as a hybridization reagent (data not shown).

Unfractionated XC cell DNA and DNA from network and supernatant fractions prepared after alkali denaturation of XC cell DNA were equally effective in annealing with 70S RNA (Fig. 8A); since over 90% of cell DNA sedimented in the network fraction, the supernatant fraction would be appreciably enriched for viral DNA if even a small number of the 20 copies of ASV DNA in this cell were not integrated. This observation is consistent with the sedimentation of viral DNA in gradients in the zonal rotor (Fig. 3).

In similar tests of DNA prepared from ASV-

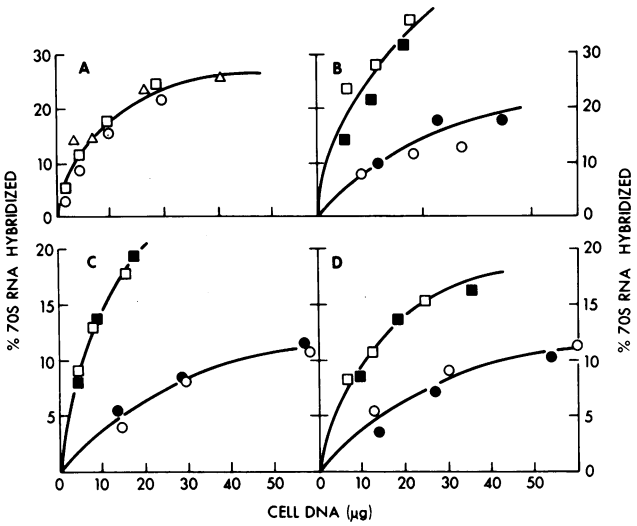

FIG. 8. *Use of the network test to detect integrated and unintegrated viral DNA in ASV-infected cells. DNA was extracted from (A) XC cells, (B) B77 ASV-transformed duck cells (passage 4 after infection), (C) duck cells infected for 22 h with B77 ASV (MOI = 6), and (D) nuclei of the acutely infected duck cells used in (C). Networks were prepared after denaturation with alkali (0.3 N NaOH, 37 C, 60 min) or heat (100 C, 5 min) and incubation at 68 C in 0.6 M NaCl for 2 h. DNA network and supernatant fractions were then treated with 0.3 N NaOH at 100 C for 20 min and ethanol precipitated; increasing amounts were annealed to [^{32}P]70S RNA and hybridization was assessed by resistance to pancreatic RNase (50 μg/ml) and T1 RNase (5 U/ml) at 37 C for 1 h in 2× SSC. The data were corrected for intrinsic resistance of the 70S RNA to RNase digestion (8 to 9%). Copy numbers were determined from calibration curves with unfractionated XC cell DNA performed in parallel with the experiments shown in each panel. (A) △, XC cell DNA; ○, XC network DNA formed after alkali denaturation; □, XC network supernatant DNA. Ninety-two percent of cell DNA entered networks. Approximately 20 copies of ASV DNA per weight of diploid cell DNA were present in all preparations. (B) ASV-transformed duck cells: network DNA (●) and supernatant DNA (■) after alkali denaturation; network DNA (○) and supernatant DNA (□) after heat denaturation. Sixty-two percent of cell DNA entered networks after alkali denaturation and 68% entered after heat denaturation. Twenty copies of ASV DNA per cell were measured in whole cell DNA, and seven copies per weight of diploid cell DNA were measured in networks. Compared with network DNA, the supernatant fractions were about sixfold enriched with viral DNA, indicating that about 14 copies were unintegrated. (C) Network DNA (●) and supernatant DNA (■) after alkali denaturation of DNA from duck cells infected 20 h with ASV; network DNA (○) and supernatant DNA (□) after heat denaturation. Sixty-four percent of cell DNA entered networks after alkali denaturation, and 63% entered after heat denaturation. A total of 1.05 copies per weight of diploid cell DNA was found in network DNAs; the supernatant fractions were sixfold enriched for viral DNA, indicating about two unintegrated copies per cell. (D) As in (C), except DNA was extracted from nuclei rather than whole cells. Sixty-nine percent entered networks after alkali denaturation, and 61% entered after heat denaturation. A total of 1.05 copies was measured per weight of diploid cell DNA in network fractions; supernatant fractions were 3.3-fold enriched, indicating about 1 copy of unintegrated DNA per nucleus.*

transformed duck cells (passaged four times at weekly intervals after infection), we found network DNA less effective than whole cell DNA in hybridization with 70S RNA, whereas supernatant DNA was about sixfold enriched for viral DNA (Fig. 8B). There was no apparent effect of the mode of denaturation upon the results. Computation of copy numbers revealed 20 copies of viral DNA per cell, with 6 copies integrated. This is considerably more than the amount of unintegrated viral DNA we observed in the alkaline sucrose gradient shown in Fig. 5. (In another unpublished gradient, about 50% of the viral DNA in such cells was unintegrated.) As discussed in the accompanying manuscript (38), the amount of unintegrated viral DNA present in these cells varies appreciably (from 1 to 15 copies per cell), although the amount of integrated DNA is relatively constant (4 to 8 copies). We are uncertain whether our earlier failure to detect unintegrated DNA in transformed duck cells reflects a lack of sensitivity of the hybridization assay employed or the absence of appreciable quantities of unintegrated DNA in the cells assessed in the earlier experiments.

We also found that the network test detected both integrated and unintegrated DNA in whole cells and nuclei after a 20-h infection (Fig. 8C and D). Again, DNA denatured by

exposure to heat or to alkali gave similar results. In all assays, about 1.0 copy of viral DNA was integrated per cell; there was a threefold enrichment of supernatant nuclear DNA and a sixfold enrichment of supernatant DNA from whole cells. This indicates that about one copy of unintegrated DNA was present in the cytoplasm, as confirmed by direct tests of DNA extracted from the cytoplasm (data not shown). The analysis by the network test is thus consistent with the alkaline gradient in the zonal rotor (Fig. 6); these results do not, however, provide an explanation for the apparent absence of unintegrated DNA at this point after infection in some earlier experiments.

DISCUSSION

Testing for integration of viral genomes. A variety of methods have been employed to determine whether viral DNA becomes covalently linked to the genome of the host cell (1, 2, 4–8, 10, 18, 23, 26, 28, 40). Although sedimentation in alkaline sucrose gradients was among the first tests applied to this issue (26), its usefulness has been compromised by the limited capacity of such gradients for extremely high-molecular-weight DNA (17, 46). The problem is compounded, of course, by the relatively small numbers of DNA copies of viral genomes that may be present in infected cells, requiring that large quantities of DNA be available for detection of viral sequences. The use of the zonal rotor circumvents this major drawback, since only one or a few gradients are necessary to permit relatively detailed analysis of small numbers of integrated and unintegrated viral DNA molecules. On the other hand, these gradients present the logistical problem of recovering DNA from large volumes for hybridization analysis in extremely small volumes. We have found, however, that high-speed sedimentation of ethanol precipitates from neutralized fractions (see above) permits excellent recovery of DNA. The amounts of labeled cell DNA recovered for analysis by hybridization usually exceeded 50% of the theoretical maximum. It might be possible to simplify the recovery of DNA by absorbing it to hydroxyapatite, benzoylated DEAE-cellulose, or nitrocellulose after neutralization; presumably it should also be possible to load the DNA directly onto nitrocellulose filters for RNA-driven hybridization.

Although more convenient tests for integration are available, they too have various shortcomings. The sodium dodecyl sulfate-NaCl fractionation originally described by Hirt for the purification of polyoma virus DNA from infected cells has also been used as a test for integration (2). In our hands, the Hirt fractionation has been very useful for the partial purification of unintegrated forms of ASV DNA (12, 34; Fig. 7), but we have found significant amounts of viral DNA trapped in the coagulum of high-molecular-weight DNA and protein. We have described and made extensive use of an assay for integration that depends upon the inclusion of integrated viral sequences in the rapidly sedimenting networks formed during brief reannealing of high-molecular-weight eukaryotic DNA (40). Although we have found excellent correlation between network tests and zonal gradient analyses in this study, the network test has some minor inherent disadvantages. A small amount (5 to 10%) of unintegrated viral DNA may be found in the pelleted networks, judging from control experiments with labeled lambda phage or SV40 DNA (36, 40); this limits the usefulness of the test in acute infections by DNA viruses. In contrast, we found no evidence of trapping of viral DNA in alkaline gradients in the zonal rotor (Fig. 2). Secondly, not all cellular DNA participates in networks, introducing some ambiguity about whether all viral DNA in a cell is integrated (discussed in more detail in references 36 and 40). Although the network test was originally performed by denaturing the DNA with heat, Bellett has recently described a modified procedure in which the DNA is denatured with alkali (4). This makes the test specific for alkali-stable integration (e.g., excluding linkage via ribonucleotides) but raises the untested possibility that partially denatured duplex circles of viral DNA might cosediment with network DNA. As illustrated here (Fig. 8), our results with the network test were not affected by the mode of denaturation.

Integration of avian RNA tumor virus-specific DNA. In this study we have tested for integration of viral DNA in several biological situations: (i) in chick cells which inherit viral genes, (ii) in virally transformed but nonpermissive mammalian cells, (iii) in transformed, virus-producing avian cells, and (iv) in acutely infected avian cells.

(i) As expected, endogenous viral genes, at least those detectable with our avian sarcoma virus-derived hybridization reagents, appear to be integrated into the chicken genome. Similar findings have been reported by Baluda and colleagues (21, 28).

(ii) Analysis in the zonal rotor demonstrates that the multiple copies of viral DNA present in cells derived several years ago from an ASV-induced rat tumor (31) are mostly, if not completely, integrated. The network test has also shown that over 90% of viral DNA is integrated in this cell line (40; Fig. 8A) and in several

other ASV-infected mammalian cells containing considerably less viral DNA (generally one to two copies per diploid cell) (34, 36, 40).

(iii) In ASV-transformed duck cells, we have found unintegrated viral DNA, in addition to the expected four to eight copies of integrated DNA by both alkaline sucrose gradient and network analyses (Fig. 5 and 8B). Since the amount of unintegrated viral DNA in transformed duck cells is variable, ranging from less than 20 to over 75% of the viral DNA per cell, it is not surprising that some earlier network tests (40) did not reveal unintegrated DNA; enrichment of the network supernatant fraction may be less than twofold, and the published measurements were probably inadequate to detect such small differences. Characterization of this unintegrated form and the mechanism of its synthesis are presented in an accompanying manuscript (38).

(iv) In acutely infected cells, integration is initiated shortly after the appearance of viral DNA in the nucleus about 6 h postinfection (35, 40) but, under the conditions of infection that we currently employ, over 75% of viral DNA may remain unintegrated 24 h after infection. In response to the sort of experiments reported here (Fig. 6 through 8), we have attempted to improve the percentage of viral DNA integrated. However, we have varied the MOI, the duration of exposure of cells to the infecting virus, and the density of the cells at the time of infection without affecting the percentage of viral DNA integrated. Reducing the MOI did, however, reduce appropriately the amount of viral DNA synthesized per cell, as previously reported (35). It is unclear whether the unintegrated viral DNA detected 1 to 3 days after infection represents precursors to circular or integrating forms or whether it consists of defective DNA that has no further role in the life cycle of the virus. Similar forms have been reported in cells infected by murine leukemia viruses (10).

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