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Virus-Specific DNA in the Cytoplasm of Avian Sarcoma Virus-Infected Cells Is a Precursor to Covalently Closed Circular Viral DNA in the Nucleus

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Three principal forms of viral DNA have been identified in cells infected with avian sarcoma virus: (i) a linear duplex molecule synthesized in the cytoplasm, (ii) a covalently closed circular molecule found in the nucleus, and (iii) proviral DNA covalently linked to high-molecular-weight cell DNA. To define precursor-product relationships among these forms of viral DNA, we performed pulse-chase experiments using 5-bromodeoxyuridine to label by density the linear species of viral DNA in the cytoplasm during the first 4 h after infection. After a 4- to 8-h chase with thymidine, a portion of the density-labeled viral DNA was transported to the nucleus and converted to a covalently closed circular form. We conclude that linear viral DNA, synthesized in the cytoplasm, is the precursor to closed circular DNA observed in the nucleus.

The ability of RNA tumor viruses to convert their single-stranded RNA genomes into double-stranded DNA is universally recognized (30, 37), but the mechanism by which this unusual form of synthesis occurs has been only partially elucidated. Three major forms of virus-specific DNA have been identified in cultured cells after productive infection by RNA tumor viruses: (i) linear duplex DNA comprised of a genome-length minus strand (8.5 to 10 kilobases, complementary to viral RNA) and segmented plus strands (0.3 to 1.5 kilobases) (10, 23, 33); (ii) covalently closed circular duplex DNA (form I) of subunit length (9, 12, 23); and (iii) viral DNA covalently integrated into host cell DNA (provirus) (35). After infection by avian sarcoma virus (ASV), the linear duplex DNA is observed in the cytoplasm within 4 h (35); form I DNA is found exclusively in the nucleus as early as 5 h after infection (12); and integration of viral DNA can occur within 9 h after infection (35). Based upon these findings, inhibitor studies (11), and analogies with other viruses (1, 2), a hypothetical scheme for the synthesis and integration of viral DNA can be constructed (Fig. 1). The linear form of viral DNA is synthesized in the cytoplasm by virus-associated, RNA-directed DNA polymerase, transported to the nucleus, and then circularized and integrated into host cell DNA, presumably with the assistance of host enzymes.

To validate this hypothesis, it is necessary to demonstrate that the cytoplasmic linear DNA is a precursor to nuclear form I DNA and that form I DNA is a precursor to integrated provirus. In this report we present evidence favoring a precursor-product relationship between cytoplasmic linear DNA and nuclear form I DNA. Viral DNA constitutes too small a fraction of total cellular DNA to permit labeling with radioactive deoxyribonucleotides, as in a traditional pulse-chase experiment. We have previously shown, however, that viral DNA synthesized in the presence of 5-bromodeoxyuridine (BUdR) has a high density attributable to the substitution of BUdR for thymidine in both strands (32, 34). We used BUdR to density label the precursor form in the cytoplasm during the first 4 h of infection by ASV; after blocking further incorporation of BUdR into viral DNA with thymidine, we followed the conversion of BUdR-substituted DNA into form I molecules in the nucleus.

MATERIALS AND METHODS

Cells and virus. We have used the QT-6 cell line (derived from a methylcholanthrene-induced fibrosarcoma of Japanese quail [21]) to study synthesis of ASV DNA (12). Cells were propagated as monolayers at 41°C in medium 199 supplemented with 10% tryptose phosphate broth, 0.1% (wt/vol) sodium bicarbonate, 5% fetal calf serum, 1% heat-inactivated chick serum, and 1% dimethyl sulfoxide (growth medium). Monolayers of QT-6 cells were infected with the B77 strain of ASV (B77-ASV) at a multiplicity of infection (MOI) of 1 focus-forming unit/cell in the presence of 4 μg of polybrene per ml. B77-ASV was grown in and titered on chicken embryo fibroblasts as previously reported (19). QT-6 cellular DNA was radioactively

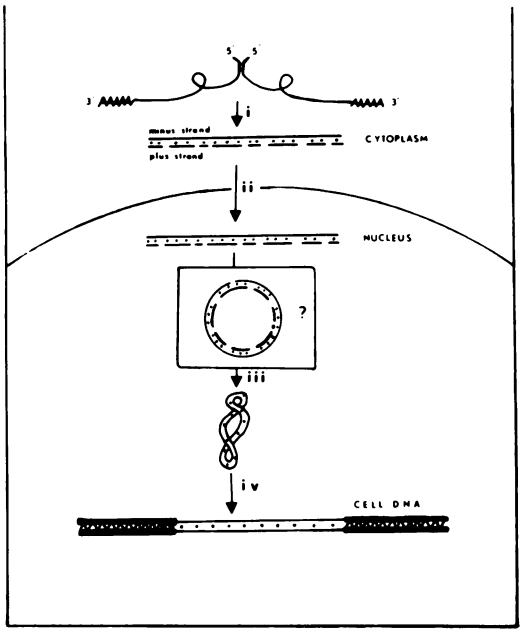

FIG. 1. *Model for the synthesis of ASV DNA. (i) The 70S viral RNA molecule is a dimer composed of two 35S subunits joined at their 5' termini (18). Synthesis of the minus strand of ASV DNA by the virion-associated RNA-dependent DNA polymerase is initiated near the 5' end of the viral genome on a $tRNA^{trp}$ molecule (5, 29). Using a terminal redundancy (3, 14, 24) in the viral RNA, the polymerase "leaps" to the 3' end of the same or a different RNA subunit and completes the synthesis of a genome-length minus strand. Before completion of the minus strand, plus-strand DNA synthesis commences (unpublished data of the authors) in the opposite direction, possibly using fragmented genome RNA as primer. (ii) The linear viral DNA molecule, synthesized in the cytoplasm and comprised of genome-length minus strands and segmented plus strands, is transported into the nucleus. (iii) Within the nucleus, the plus-strand fragments are completed and joined (possibly by cellular enzymes) immediately before the molecule is covalently closed. This conversion may occur via an as yet unobserved open circular intermediate. (iv) Covalently closed circular DNA presumably integrates covalently into the cellular genome at unknown sites by a recombinational event (1). To allow synthesis of genomic viral RNA directly from a template of proviral DNA, at least one copy must be oriented so that it is colinear with the viral RNA.*

labeled by the inclusion of 0.1 μCi of [^{3}H]- or [^{14}C]-thymidine per ml in normal growth medium. To label QT-6 cellular DNA radioactively in the presence of BUdR, [^{3}H]deoxyguanosine (0.1 to 0.4 μCi/ml) was included in the normal growth medium.

BUdR labeling and wash procedure. To label DNA with BUdR, QT-6 cells were grown in normal growth medium supplemented with 10 μg of BUdR (Calbiochem) per ml. To stop BUdR incorporation, the cells were washed twice with Tris-glucose (0.14 M NaCl, 5 mM KCl, 5.5 mM glucose, and 25 mM Tris-hydrochloride, pH 7.4) containing 50 μg of thymidine per ml and then incubated in normal growth medium containing 50 μg of thymidine per ml.

Cell fractionation and DNA extractions. Cells were fractionated into nucleus and cytoplasm after disruption in 1% Nonidet P-40 (Shell Oil) as previously described (12). The nuclei were then dissolved at 1×10^7 per ml in 20 mM Tris (pH 7.2)-10 mM EDTA (TE buffer) and lysed by the addition of sodium dodecyl sulfate (SDS) to 1%, and chromosomal DNA was precipitated by the addition of 5 M NaCl to 1 M according to the method of Hirt (15). After overnight incubation at 4°C, the chromosomal DNA was pelleted by centrifugation of 13,000 × *g* for 30 min. DNA was prepared from the cytoplasm and the SDS-NaCl supernatant by conventional methods (32, 35). In brief, the samples were incubated with self-digested Pronase (500 μg/ml) at 37°C for 1 h followed by phenol extraction and ethanol precipitation. The DNA was resuspended in TE buffer and digested for 1 h with pancreatic RNase (100 μg/ml) at 37°C followed by Pronase digestion (250 μg/ml) at 37°C for 30 min, phenol extraction, and ethanol precipitation.

Gradient sedimentation. Fractionation of DNA according to the extent of substitution of BUdR for thymidine was accomplished by equilibrium banding in density gradients of CsCl. Samples of DNA in TE buffer were sheared by three passages through a 19-guage needle and mixed with solid CsCl (Harshaw radiotracer grade) to give a final density of 1.75 g/cm^3. Centrifugation was for 60 h at 20°C in either the type 40 rotor (at 33,000 rpm) or the type 42.1 rotor (Beckman) (at 31,000 rpm), depending on the number of cells extracted. Densities were determined both from measurements of refractive index (with a Bausch and Lomb refractometer) and from the banding position of radiolabeled cellular DNA.

Covalently closed circular DNA (form I) was separated from relaxed forms (linear or nicked circular forms III and II) by banding in CsCl density gradients containing intercalating dyes. Each gradient contained 300 μg of ethidium bromide (EB) (22) or propidium diiodide (PI_2) (16) per ml, and the sample was dissolved in TE buffer to give final densities of 1.62 g/cm^3. The gradients were centrifuged for 60 h at 33,000 rpm in a type 40 rotor at 20°C.

Covalently closed circular DNA was also separated from open circular or linear forms by sedimentation in alkaline sucrose gradients (35). Gradients of 5 to 20% sucrose containing 0.3 N NaOH, 0.7 N NaCl, and 0.001 M EDTA were prepared in polyallomer tubes and centrifuged at 17,000 rpm for 16 h at 20°C in an SW27.1 rotor (Beckman). ^{3}H-labeled plasmid DNA, pML21 (forms I and II; molecular weight, 6.7×10^6), served as an external sedimentation marker.

Hybridization reagents. ^{32}P-labeled complementary DNA (cDNA) was prepared in vitro from detergent-activated B77-ASV in the presence of actinomycin D as described (8). The product synthesized was fractionated into single- and double-stranded fractions on hydroxyapatite, and the single-stranded fraction was used for annealing. This ^{32}P-labeled cDNA, which

contained sequences primarily complementary to the 5′ end of the viral genome (6, 13), had a specific activity of 1×10^8 cpm/μg.

^{125}I-labeled ASV RNA was prepared as previously described by Commerford (4) and had a specific activity of 1×10^8 cpm/μg.

Hybridization assay for ASV specific DNA. Hybridization of labeled ASV cDNA to unlabeled viral DNA was performed as previously described (12). After the addition of 100 μg of calf thymus DNA as carrier, samples were diluted to 1.0 ml in 0.3 N NaOH, heated to 80°C for 2 h (to fragment and denature the DNA), neutralized, ethanol precipitated, and resuspended in 20 μl of TE buffer. After addition of 1,000 to 1,500 cpm of [^{32}P]cDNA and adjustment of the NaCl concentration to 0.6 M, the samples were overlayered with mineral oil and incubated at 68°C for 60 h. Annealing of the cDNA was assayed by resistance to the single-strand-specific nuclease S1 (27). The amount of unlabeled viral DNA in each sample was then calculated from a calibration curve constructed in parallel with each hybridization experiment by annealing ^{32}P-labeled cDNA to increasing amounts of DNA from XC cells (rat cells transformed by the Prague C strain of ASV) (28). Since the standard used (DNA from XC cells) is double stranded, the calibration is accurate only when both strands of the viral DNA are present. When only one strand of viral DNA is present, i.e., at the top of an alkaline sucrose gradient where the long minus strands are separated from the short plus strands, this calibration cannot be used.

[^{125}I]RNA was used to detect the minus strand of viral DNA. Annealings with [^{125}I]RNA were performed exactly as described above for [^{32}P]cDNA except that 100 μg of yeast RNA carrier per ml was added to each reaction. The annealing of [^{125}I]RNA was assayed by resistance to digestion by a mixture of pancreatic and T1 RNase (50 μg/ml, 5 U/ml) in 2 × SSC (SSC = 0.15 M NaCl + 0.015 M sodium citrate) at 37°C for 1 h. The nuclease resistance (ca. 5%) of the cDNA and RNA after incubation with calf thymus DNA was considered background for the assay and was subtracted from each analysis.

RESULTS

BUdR labeling of viral DNA. We have previously shown (32, 34) that viral DNA synthesized in duck embryo fibroblasts infected with ASV can be density labeled with BUdR. When cells have been labeled for an appropriate time, viral DNA that is fully substituted with BUdR (HH DNA) can be separated from partially substituted (HL) and unsubstituted (LL) cellular DNA by banding in CsCl density gradients.

We used QT-6 cells in this study because they support three- to fivefold more viral DNA synthesis than duck embryo fibroblasts after infection with B77-ASV (12). In addition, one to three copies of form I viral DNA are regularly synthesized per cell after infection of QT-6 cells with B77-ASV at high MOI (12). To document our ability to density label viral DNA in the QT-6 line, cells were infected with B77-ASV (MOI = 1) in the presence of 10 μg of BUdR and 0.1 μCi of [^{3}H]dG per ml. After 4 h the cells were fractionated by the SDS-NaCl precipitation of Hirt (15), and DNA was extracted from the supernatant fraction and banded to equilibrium in a CsCl gradient. The position of LL and HL cellular DNA in the gradient was determined from the ^{3}H radioactivity within a portion of each fraction, and viral DNA was detected by annealing a portion of each fraction of the gradient with virus-specific [^{32}P]cDNA, as described in Materials and Methods (Fig. 2). All the viral DNA banded at a density of ca. 1.78 g/cm^3, consistent with the replacement of 80 to 100% of thymidine residues by BUdR in both strands during infection of QT-6 cells, as in duck embryo fibroblasts. Cellular DNA banded at densities of 1.70 and 1.74 g/cm^3, appropriate for LL and HL DNAs.

Efficacy of chasing BUdR from QT-6 cells. To use BUdR labeling in a pulse-chase experiment, we needed to document our ability to block incorporation of BUdR into DNA during the chase portion of the experiment. Weintraub (38) has shown that incorporation of BUdR into chicken embryo fibroblast DNA is decreased by 90% within 1 to 2 min by washing the cells in medium containing thymidine. We therefore designed a chase procedure in which QT-6 cells were washed twice in Tris-glucose buffer containing 50 μg of thymidine per ml and then incubated in growth medium containing 50 μg of thymidine per ml.

We first assessed the effect of this chase procedure on the labeling of cellular DNA under our experimental conditions. Cells were infected with B77-ASV in the presence of 10 μg of BUdR per ml. One set of cells was labeled from 2 to 4 h after infection with [^{3}H]dG and harvested 4 h postinfection; this procedure might be expected to generate ^{3}H-labeled HL cellular DNA. Four hours after infection, two other sets of cells were washed and incubated with medium containing 50 μg of thymidine per ml as described above. One of these sets was labeled with [^{3}H]dG for 15 min immediately after the wash, and the other set was labeled from 15 to 30 min after the wash. After extraction and shearing, the DNA from all three sets was banded to equilibrium in CsCl gradients containing an internal ^{14}C-labeled marker of LL QT-6 DNA. As expected, the [^{3}H]DNA radiolabeled in the presence of BUdR banded at the position of HL DNA (1.74 g/cm^3) (Fig. 3A), whereas the average density of DNA synthesized 0 to 15 min after the chase was only slightly greater than that of LL DNA (Fig. 3B); all the DNA synthesized 15 to 30 min after the chase co-banded with the ^{14}C-labeled LL DNA (Fig. 3C), indicat-

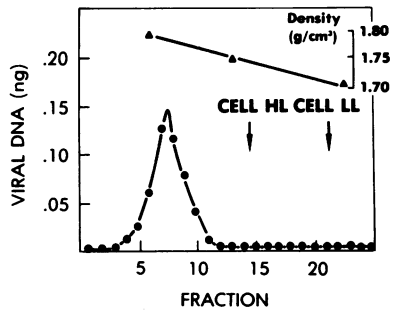

FIG. 2. *BUdR density labeling of viral DNA in QT-6 cells. 2 × 10⁶ QT-6 cells were infected with B77-ASV (MOI = 1) in the presence of 10 μg of BUdR, 4 μg of polybrene, and 0.1 μCi of [³H]dG per ml. Four hours after infection the cells were trypsinized, washed twice in Tris-glucose, and lysed by the SDS-NaCl procedure described by Hirt (15). DNA extracted from the SDS-NaCl supernatant was centrifuged to equilibrium in a CsCl density gradient at 33,000 rpm in a type 40 rotor for 60 h at 20°C. After collection from below, 10% of each fraction was counted directly to determine the banding position of ³H-labeled LL and HL cellular DNA. Densities were determined from the refractive index. After denaturation and fragmentation by incubation at 80°C in 0.3 N NaOH, the sample was precipitated with ethanol, and viral DNA was detected by annealing ³²P-labeled ASV-specific cDNA (1,000 cpm) to the remainder of each fraction as described in Materials and Methods. Annealing was assessed by resistance of the cDNA to S1 nuclease digestion, and the amount of viral DNA was determined by comparison to a calibration curve generated by annealing the [³²P] cDNA to increasing amounts of XC DNA as described in Materials and Methods.*

ing that the chase was completely effective within 15 min.

Although the chase procedure appeared highly effective with respect to cellular DNA, viral DNA, synthesized in the cytoplasm by a viral polymerase, might possibly utilize different nucleotide pools. We therefore also tested our ability to prevent BUdR incorporation into viral DNA. Uninfected QT-6 cells were incubated for 4 h with 10 μg of BUdR per ml, washed twice, and then infected with B77-ASV in the presence of 50 μg of thymidine per ml. After 8 h, viral DNA extracted from the SDS-NaCl supernatant was analyzed in a CsCl equilibrium density gradient (Fig. 4). All viral DNA detected by annealing with ³²P-labeled cDNA banded in the region of LL DNA, indicating that our wash procedure was effective in blocking BUdR incorporation into viral DNA. Since viral DNA synthesis is detectable within 1 h after infection under these conditions (unpublished data of V. Smith and authors), the chase procedure rapidly stops BUdR incorporation into viral DNA. However, it was not possible to examine the kinetics of the chase procedure for viral DNA as stringently as for cellular DNA.

Pulse-chase experiments with density-labeled viral DNA. (i) Linear viral DNA is precursor to form I DNA. To test and illustrate our experimental plan without the complexities introduced by cell fractionation, we per-

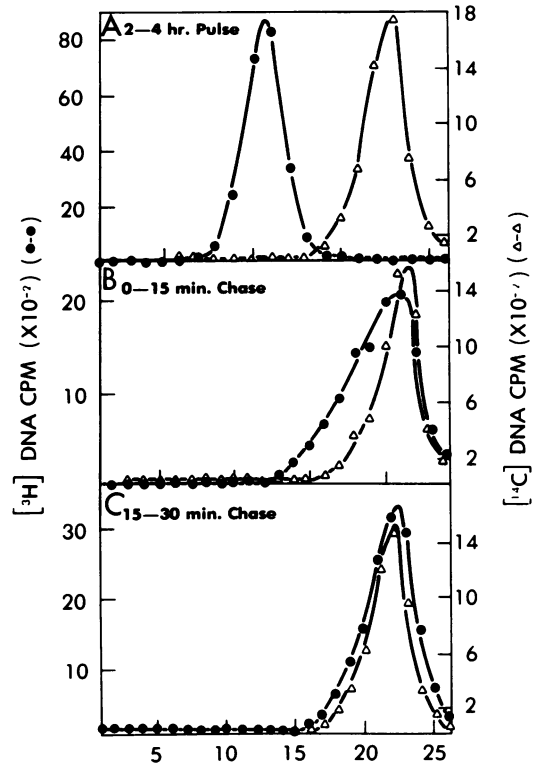

FIG. 3. *Efficiency of chase with QT-6 cellular DNA. Three sets of QT-6 cells were infected with B77-ASV (MOI = 1) in the presence of 10 μg of BUdR and 4 μg of polybrene per ml. Two hours after infection, one set of cells (A) was labeled with 0.2 μCi of [³H]dG per ml in the presence of BUdR. Four hours after infection, set A was harvested and total cellular DNA was extracted. At the same time, the remaining two sets of cells (B and C) were washed twice in Tris-glucose containing 50 μg of thymidine and incubated in growth medium containing 50 μg of thymidine per ml. The cells in set B were labeled from 0 to 15 min after the wash with 0.4 μCi of [³H]dG per ml, and the other cells (C) were labeled with [³H]dG from 15 to 30 min after the wash. Cellular DNA extracted from each set of cells was sheared and banded to equilibrium in CsCl density gradients, as described in the legend of Fig. 2, after the addition of ¹⁴C-labeled unsubstituted QT-6 cell DNA. The gradient fractions were counted directly in an aqueous scintillation fluor to determine the banding position of ³H (●)- and ¹⁴C (△)-labeled cellular DNA.*

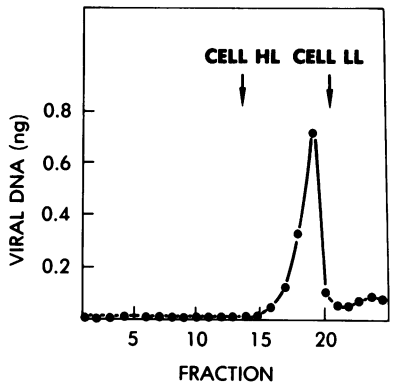

FIG. 4. *Efficiency of chase with viral DNA. QT-6 cells were incubated for 4 h with 10 μg of BUdR and 0.1 μCi of [^{3}H]dG per ml. The cells were then washed twice with Tris-glucose containing 50 μg of thymidine per ml and infected with B77-ASV (MOI = 1) in the presence of 4 μg of polybrene and 50 μg of thymidine per ml. Eight hours after infection, the cells were harvested, and the density of viral DNA in the SDS-NaCl supernatant was determined by banding in a CsCl density gradient as described in the legend of Fig. 2.*

formed the preliminary experiment illustrated in Fig. 5. Our previous results (12) demonstrated that little or no form I viral DNA would be present 4 h after infection. Our experiment was therefore designed to density label the linear form of viral DNA during the first 4 h after infection, wash out the BUdR, and determine whether density-labeled DNA was converted into form I molecules.

QT-6 cells were infected with B77-ASV in the presence of BUdR and 0.1 μCi of [^{3}H]dG per ml; after 4 h, a portion of the cells (pulse sample) was harvested, and a second portion (chase sample) was subjected to the thymidine chase procedure and allowed to incubate for an additional 4 h before collection. Viral DNA, extracted from the SDS-NaCl supernatant of the pulse sample, banded in the position expected for HH DNA in a CsCl equilibrium density gradient (Fig. 5A) and in the position expected for linear DNA when rebanded in a CsCl density gradient containing propidium diiodide (CsCl-PI_2) (Fig. 5C). (Binding of PI_2, a buoyant intercalating dye, is restricted in superhelical [form I] DNA compared with linear or nicked circular molecules, causing form I DNA to exhibit a greater density than do the other forms in CsCl-PI_2 gradients.)

In contrast, viral DNA extracted from the SDS-NaCl supernatant of the chase sample exhibited a broad spectrum of densities after equilibrium centrifugation in CsCl gradients (Fig. 5B). The DNA ranged from HH molecules presumably completed during the 4-h labeling with BUdR to LL molecules synthesized only during the chase period; the species of intermediate density were presumably molecules of different "ages," dating from the time of the thymidine chase. The heterogeneous density of viral DNA observed after the thymidine chase contrasted sharply with the homogeneity of cellular DNA synthesized after a similar chase (Fig. 3B and C). Although it is possible that the chase procedure is less efficient with respect to viral DNA, the observed data are also consistent with the hypothesis that viral DNA is elongated at a relatively slow rate. This would mean that many incomplete molecules were present 4 h after infection and completed during the chase; this idea is supported by kinetic analysis of the synthesis of viral DNA (see Discussion).

When the HH DNA recovered from the chase sample was centrifuged in CsCl-PI_2 gradients (Fig. 5D), approximately 25% of the viral DNA banded in the position of form I DNA, demonstrating that linear DNA synthesized during the first 4 h of infection could be found in a covalently closed circular form 4 h later.

(ii) Linear viral DNA in the cytoplasm is precursor to form I DNA in the nucleus. To determine whether the linear precursor to form I DNA was present initially in the cytoplasm of infected cells and then transported into the nucleus, a pulse-chase experiment including cell fractionation was performed. The DNAs from the nuclear and cytoplasmic fractions of the pulse and chase cells were banded to equilibrium in CsCl density gradients, and the position of cellular DNA and viral DNA was detected as described previously (legend to Fig. 2). All of the viral DNA present in the 4-h pulse samples banded at the position of HH DNA (Fig. 6A and C). A small amount of viral DNA (less than 2% of the total) was detected in the nuclear fraction 4 h after infection and probably reflected contamination of the nuclei with cytoplasmic material, since the nuclei were not washed after cell fractionation.

Both the nucleus and cytoplasm from the chase cells contained viral DNA banding in a broad spectrum of densities (Fig. 6B and D), in agreement with the previous experiment (Fig. 5B). The density of each molecule presumably reflects the degree of its completion at the time of the chase procedure; therefore the gradient of densities corresponds to a gradient of ages of viral molecules. Most if not all of the oldest (HH) viral DNA in the nucleus must have been fully synthesized in the cytoplasm during the pulse of BUdR and transported to the nucleus during the chase period, since there was at least five times more HH DNA in the nuclei at 8 h than at 4 h after infection. Similarly, the mole-

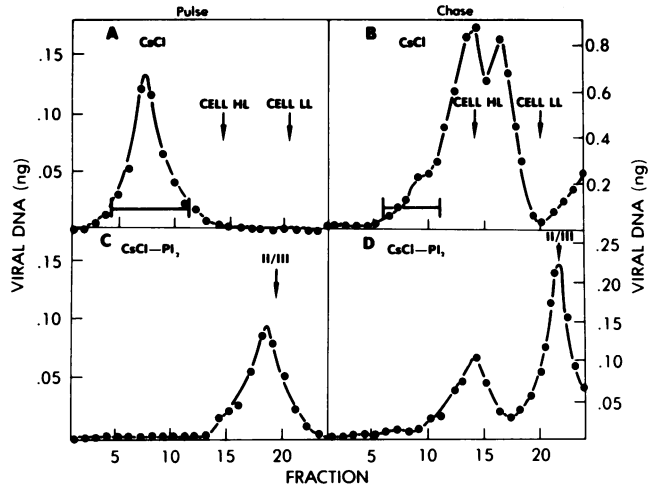

FIG. 5. *Analysis of pulse-labeled viral DNA in CsCl and CsCl-PI_2 gradients. Two sets of QT-6 cells (7×10^6 each) were infected with B77-ASV (MOI = 1) in the presence of 10 μg of BUdR, 4 μg of polybrene, and 0.1 μCi of [^{3}H]dG per ml. Four hours after infection, the pulse cells were trypsinized and subjected to an SDS-NaCl fractionation; the chase cells were washed as described in the legend to Fig. 3 and incubated in growth medium containing 50 μg of thymidine per ml. Eight hours after infection, the chase sample was harvested as described for the 4-h sample. After DNA extraction, the pulse and chase samples were centrifuged to equilibrium in CsCl density gradients as described in the legend of Fig. 2. Viral DNA was detected by annealing 20% of each gradient fraction with ^{32}P-labeled ASV cDNA. The position of HL and LL cellular DNA, indicated by the arrows, was determined by directly counting 1% of each gradient fraction. Pools of HH viral DNA, indicated by the bars, were made, and the DNA was recovered by precipitation with ethanol. The secondary structure of the viral DNA was then assayed by banding the DNA from each pool in CsCl density gradients containing the intercalating buoyant dye PI_2. One-third of the HH DNA recovered from the pulse and chase samples was banded to equilibrium in a CsCl gradient (starting density of 1.62 g/cm^3) containing 300 μg of PI_2 per ml. The density at which open circular (form II) or linear (form III) HH DNA would band is indicated by the arrow. The CsCl-PI_2 gradients were centrifuged for 60 h at 33,000 rpm in a type 40 rotor at 20°C. Viral DNA was detected in the CsCl-PI_2 gradients by annealing with ^{32}P-labeled cDNA after denaturation and ethanol precipitation as described in the legend to Fig. 2.*

cules of intermediate density were at least initiated in the cytoplasm during the pulse period. However, the site at which these molecules were completed and the site of synthesis of the youngest (LL) molecules found in the nucleus after the chase cannot be determined from this experiment. Assuming that all viral DNA synthesis is initiated in the cytoplasm, the presence of LL viral DNA molecules in the nucleus at times when HH and HL molecules are present in the cytoplasm (Fig. 6B and D) implies that transport of viral DNA into the nucleus is not dependent on the age of the molecules.

Fractions from the indicated regions of the CsCl gradients shown in Fig. 6A through D were pooled, and the structure of DNA in each pool was analyzed by centrifugation in CsCl-EB gradients (Fig. 7). In accord with our previous results (12), no form I viral DNA was detectable in the cytoplasm at either 4 or 8 h after infection (Fig. 7A). In addition, none of the small amount of viral DNA associated with the nucleus at 4 h postinfection was form I DNA (Fig. 7B); as noted earlier, the small amount of DNA present in the nuclear fraction at 4 h probably represented cytoplasmic contamination. By 8 h after infection, when there was five times more HH DNA in the nucleus than at 4 h (Fig. 6C and D), approximately 50% of the HH DNA in the nucleus consisted of form I molecules (Fig. 7C). Similar analysis in CsCl-EB gradients of DNA recovered from the HL region of the CsCl gradient shown in Fig. 6D indicated that 5 to 10% consisted of form I molecules (data not shown). These results indicate that the majority of form I viral DNA molecules present in the nucleus must have come from the pool of BUdR-substituted cytoplasmic viral DNA. Since we encountered significant variability in recovering BUdR-substituted DNA from the preparative CsCl gradients (Fig. 6), our conclusions are based upon the proportions of form I DNA in the recovered DNA (Fig. 7) and not upon the absolute amounts of form I DNA.

Since the density of DNA used in these experiments was altered by BUdR substitution as well as by the binding of intercalating dyes, we confirmed our observations by sedimentation in

alkaline sucrose gradients, a method independent of the density of DNA. In alkaline sucrose gradients, form I molecules partially denature and collapse into a fast-sedimenting form (36), permitting both the detection of form I molecules and an estimation of their size (26). Genomelength strands derived from linear molecules should sediment as 18 to 20S in these gradients (26); since there is no evidence for multimeric linear molecules, viral DNA migrating faster than 20S was presumed to be convalently closed circles. In addition, these gradients permit measurement of the size of each strand present in the linear viral DNA molecules (see below).

HH viral DNA from the various pools shown in Fig. 6 was sedimented in alkaline sucrose gradients and assayed by annealing with [^{32}P]-cDNA (Fig. 8). These gradients confirmed the observations made by equilibrium density centrifugation in CsCl-EB gradients (Fig. 7). There was no evidence of rapidly sedimenting (form I) DNA in the cytoplasm at either 4 or 8 h. (Insufficient viral DNA was recovered from the 4-h sample of nuclear DNA to allow analysis in an alkaline sucrose gradient). The HH sample from the nuclear fraction after 8 h of infection contained two populations of form I viral DNA. The large population sedimented at 65S, consistent with a molecular weight of about 6.5 × 10^6. The other component sedimented at around 45S, consistent with a molecular weight of about 2 × 10^6 to 3 × 10^6. This smaller population presumably represents the highly defective form I viral DNA we have previously observed in QT-6 cells infected with B77-ASV (12). Although cDNA annealed much more extensively to linear DNA at the top of these gradients than to the closed circular DNA, the relative proportions of form I and linear DNA cannot be estimated from these analyses. The efficiency of annealing of cDNA to plus strands from linear DNA was exaggerated because the minus strands, which normally compete with the cDNA, were separated from the plus strands on the basis of size. This accounts for the apparent

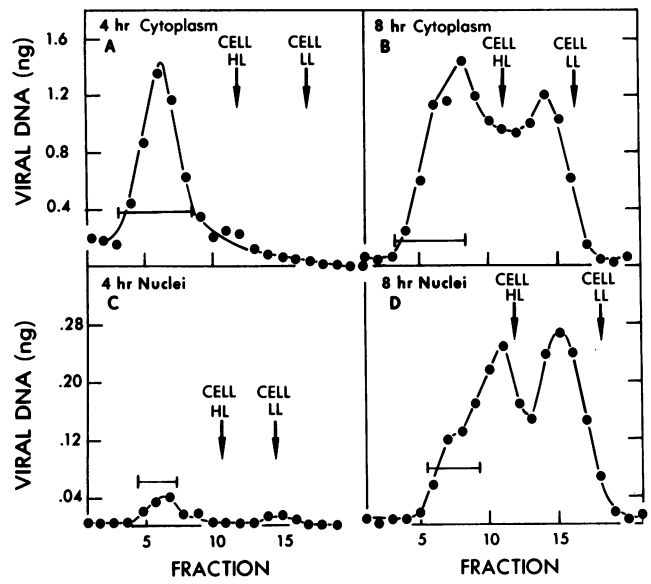

FIG. 6. *CsCl gradient analysis of pulse-labeled viral DNA in the nucleus and cytoplasm. QT-6 cells (2 × 10^6) were prelabeled for 24 h with 0.1 μCi of [^{3}H]thymidine per ml. Cells were then infected with B77-ASV (MOI = 1) in the presence of 10 μg of BUdR per ml as described in Materials and Methods. Four hours after infection, half the cells were harvested and the other half were subjected to the chase procedure and incubated for an additional 4 h in growth medium containing 50 μg of thymidine per ml. The cells were fractionated into nuclei and cytoplasm; DNA was prepared from the cytoplasm, and an SDS-NaCl supernatant was prepared from the nuclei as described in Materials and Methods. The DNA was then banded to equilibrium in CsCl density gradients in a type 42.1 rotor, and viral DNA was detected by annealing 10% of each fraction with ^{32}P-labeled cDNA as described in the legend to Fig. 2. The positions of the cellular HL and LL DNA, indicated by the arrow, were determined by directly counting ^{3}H in 1% of each gradient fraction. The pools of HH DNA indicated by the bars were made, and DNA was recovered by precipitation with ethanol and analyzed in CsCl-EB gradients (Fig. 7) or alkaline sucrose gradients (Fig. 8).*

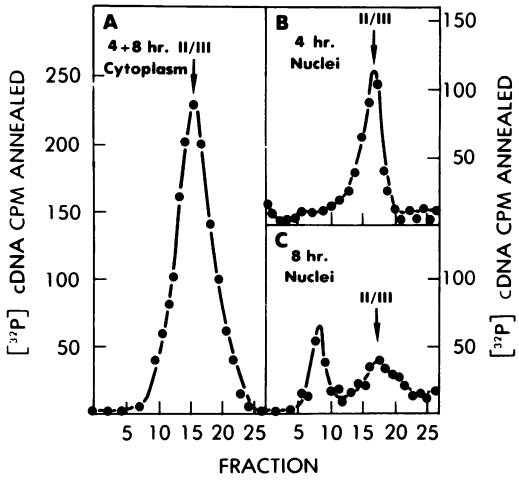

FIG. 7. *Analysis of pulse-labeled viral DNA in CsCl-EB gradients. HH DNA from the pools indicated in Fig. 6 was concentrated by precipitation with ethanol and banded in CsCl-EB density gradients (starting density = 1.62 g/cm). The 4- and 8-h cytoplasmic samples were pooled to reduce the number of analyses, since previous data (12) indicated the absence of form I DNA from the cytoplasm. The arrow indicates the banding position of linear (III) or open circular (II) DNA. Five percent of the cytoplasmic pool was analyzed, whereas all of the 4-h nuclear and one-third of the 8-h nuclear pools were analyzed. Viral DNA was detected by annealing each fraction of the gradients with [^{32}P]cDNA as described in Materials and Methods.*

discrepancy between Fig. 7C and 8B.

Size of the strands of viral DNA present in linear forms. We have reported that the linear viral DNA present in the cytoplasm of ASV-infected duck cells contains a long minus strand (complementary to the viral genome) and a short plus strand (same polarity as the viral genome) (31). The same form of viral DNA is present in ASV-infected QT-6 cells (H. E. Varmus, S. Heasley, H. J. Kung, H. O. Oppermann, V. C. Smith, J. M. Bishop, and P. R. Shank, submitted for publication), and similar forms of viral DNA have been found in murine leukemia virus-infected cells (10) and in mouse mammary tumor virus-infected cells (23). This unusual linear duplex DNA appears to be the characteristic form of DNA transcribed in vivo from the genome of RNA tumor viruses.

We therefore designed a pulse-chase experiment with the BUdR density-labeling protocol to determine whether elongation of the plus-strand fragments was detectable during the chase. Viral DNA recovered from the HH region of CsCl density gradients from the cytoplasm after a 4-h pulse with BUdR and from both the nucleus and cytoplasm after an additional 8-h chase with thymidine (cf. Fig. 6) was sedimented in alkaline sucrose gradients (Fig. 9). Minus-strand viral DNA extracted from both the 4-h and 12-h cytoplasm and the 12-h nuclei, detected by annealing with ^{125}I-labeled viral RNA, sedimented at 10 to 20S (Fig. 9). The plus strand,

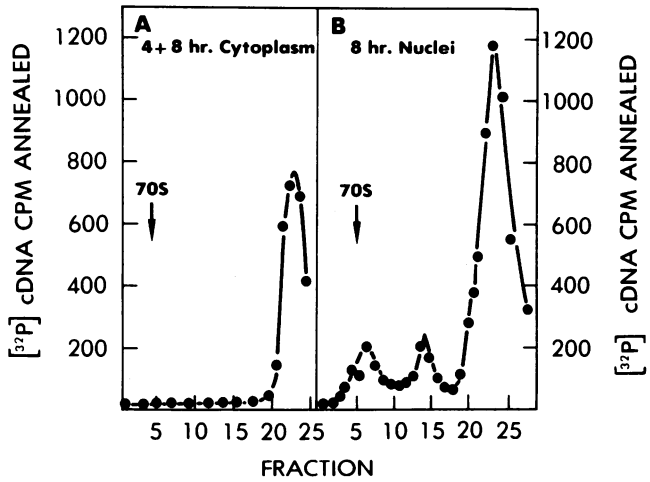

FIG. 8. *Analysis of pulse-labeled viral DNA in alkaline sucrose gradients. DNA recovered from the HH pools shown in Fig. 6 was sedimented in alkaline sucrose gradients at 17,000 rpm for 16 h in an SW27.1 rotor at 20°C. Two-thirds of the nuclear pool and one-twentieth of the cytoplasmic pool were analyzed. A parallel gradient contained ^{3}H-labeled plasmid DNA (pML21) as a sedimentation marker. (pML-21 is a bacterial plasmid that exists as a form I DNA molecule of 6.7 × 10^{6} daltons and has a sedimentation coefficient of 70S in alkaline sucrose.) Sedimentation was from right to left. Viral DNA present in each gradient fraction was detected by annealing with ^{32}P-labeled cDNA as described in Materials and Methods.*

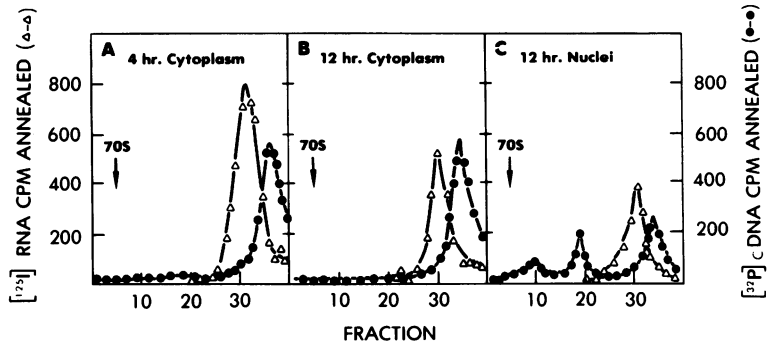

FIG. 9. *Analysis of both strands of pulse-labeled viral DNA in alkaline sucrose gradients. HH viral DNA was recovered from CsCl density gradients of the nucleus and cytoplasm of QT-6 cells infected and density pulse labeled as described in Fig. 6, except that the chase was extended until 12 h after infected to increase the amount of HH DNA transported into the nucleus. The HH DNA samples from the cytoplasm of 1 × 10⁸ cells and from the SDS-NaCl supernatant of the nuclei from 5 × 10⁸ cells were sedimented in an alkaline sucrose gradient for 16 h at 17,000 rpm in an SW27.1 rotor at 20°C. Minus-strand DNA was detected by annealing to ^{125}I-labeled viral RNA, and plus-strand DNA was detected by annealing to ^{32}P-labeled cDNA. To reduce the number of analyses (since any rapidly sedimenting [form I] DNA must contain both strands of viral DNA), fractions 1 to 20 were analyzed with [^{32}P]cDNA, whereas fractions 21 to 40 were divided in half and analyzed with each reagent. A parallel gradient contained the ^{3}H-labeled pML21 DNA (form I) as a sedimentation marker. Sedimentation was from right to left.*

detected by annealing with [^{32}P]cDNA, sedimented at 4 to 8S in both cytoplasmic samples (Fig. 9A and B). Furthermore, the plus strands present in the nucleus 12 h after infection sedimented at 4 to 8S unless they were present in form I molecules (Fig. 9C). Therefore we are unable to detect plus-strand intermediates between the short plus strands in the linear molecule and the full-length plus strands present in form I molecules.

DISCUSSION

We wish to draw one central conclusion from the experiments presented in this report: the linear form of viral DNA, synthesized in the cytoplasm of cells infected with ASV, is a precursor to the closed circular form found in the nucleus a few hours later. We have established this relationship by the use of the traditional pulse-chase protocol, which has been used in many contexts to illustrate precursor-product relationships. Although pulse-chase experiments most commonly use labeling of precursor candidates with radioactive isotopes, the small quantities of viral DNA in RNA tumor virus-infected cells obliged us to use density labeling with BUdR to identify precursor DNA, which was then detected by molecular hybridization. Using this protocol, we have shown that a significant proportion of the density-labeled linear viral DNA can be chased into form I molecules (Fig. 5–9) and that this DNA is also transported from the nucleus into the cytoplasm during the chase period (Fig. 6).

We encountered significant variability in the recovery of the small amounts of viral DNA from the preparative CsCl gradients, thereby impairing our ability to account for all of the viral DNA in subsequent analyses. On the other hand, since there was five times more HH DNA in the nucleus after the chase (Fig. 6) and over 50% of the HH DNA in the nucleus after the chase was in the form I configuration (Fig. 7C), we conclude that the majority of the form I DNA in the nucleus was synthesized as linear molecules in the cytoplasm. Taken together, these observations support steps i to iii in the proposed scheme for the synthesis of viral DNA (Fig. 1).

The results presented here do not exclude the possibility that the cytoplasmic form of viral DNA is an open circle rather than a linear molecule. Using agarose gel electrophoresis and analysis with restriction endonucleases, we have established that the cytoplasmic viral DNA molecule is linear (Shank et al., in preparation). Similar results have been observed with the DNA of mouse mammary tumor virus (Shank et al., in preparation); in addition, the open forms of Moloney murine leukemia virus, mouse sarcoma virus, and spleen necrosis virus DNAs are in linear rather than open circular conformation (3, 7, 23).

In view of the evidence for the efficiency of our chase procedure (Fig. 3 and 4), we were surprised to find that a large proportion of viral DNA exhibited the density of partially labeled molecules after the chase period (Fig. 5C and

D; Fig 6C and D). One obvious interpretation of this result is that many of the viral DNA molecules initiated during the first 4 h after infection were not complete at that time, and that their completion during the chase period conferred an intermediate density upon them. We have recently obtained corroborating evidence for this view. Studies of the kinetics of synthesis of viral DNA (under conditions identical to those used in this report) have revealed that minus strands are slowly elongated, requiring 3 to 6 h for completion, and synthesis of plus strands is retarded about 1 to 2 h relative to synthesis of minus strands (Varmus et al., submitted). Thus, at the time of our chase procedure, only a minority of DNA molecules were likely to be completed duplexes which would retain their HH density during the chase procedure. It was, however, impossible for us to lengthen our pulse period, since effective demonstration of the precursor-product relationship demanded that little or no nuclear DNA be detectable at the conclusion of the BUdR labeling period.

Although it is apparent from our results that at least a portion of the cytoplasmic DNA migrates to the nucleus and enters a closed circular form, we do not know whether all of the observed cytoplasmic DNA is capable of these transitions. The large amount of HH DNA that persisted in the cytoplasm during the chase (Fig. 6B) might have represented incomplete molecules that could not be transported into the nucleus. Previous results from our laboratory and others indicate that viral DNA is synthesized in considerable excess over the integrated species (12, 17) and that some of this excess appears to remain in the cytoplasm (36). In addition, we and others have shown that a significant portion of the unintegrated linear DNA is less than genome length (12, 25). These short species could represent abortive transcription products, precursors to full-length forms, and/or precursors to the small species of closed circular viral DNA we have previously described (12).

Although our experiments provide direct evidence for the precursor-product relationship between the cytoplasmic linear and the covalently closed nuclear forms of viral DNA, they do not address the question of whether the covalently closed form is the precursor to the integrated provirus (step iv, Fig. 1). In addition, they do not reveal the molecular nature of the circularization process. Although the simplest model for circularization of the viral DNA would involve single-stranded regions ("sticky ends") at the termini of the linear molecules, such ends have not yet been demonstrated, and recombinational mechanisms of circularization remain equally plausible.

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