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Unintegrated Viral DNA Is Synthesized in the Cytoplasm of Avian Sarcoma Virus-Transformed Duck Cells by Viral DNA Polymerase

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We have examined the location, structure, and mechanism of synthesis of unintegrated viral DNA present in fully transformed cultures of avian sarcoma virus-infected duck cells. De novo synthesis of the unintegrated forms several weeks after the initial infection was documented by labeling unintegrated DNA in both strands with 5-bromodeoxyuridine. The unintegrated DNA is synthesized in, and probably confined to, the cytoplasm, and it consists of duplexes of short "plus" strands (ca. 0.5×10^6 to 1.0×10^6 daltons) and "minus" strands the length of a subunit of the viral genome (ca. 2.5×10^6 to 3.0×10^6 daltons). The structure of the duplex and the mode of incorporation of density label support the hypothesis that the unintegrated DNA is synthesized from an RNA template by virus-coded DNA polymerase.

In the accompanying paper, we reported that unintegrated viral DNA can be detected in avian sarcoma virus (ASV)-transformed duck cells by analysis of DNA in alkaline sucrose gradients in a zonal rotor or by assay of viral DNA not included in networks of high-molecular-weight cell DNA (6). The quantity of unintegrated DNA present in cells many generations after the initial infection indicated that new synthesis of this DNA probably occurs in the transformed cells; the "plus" and "minus" strands are similar in size to those in the predominant species of viral DNA found early after infection (4a, 6; R. V. Guntaka, O. C. Richards, P. R. Shank, J. M. Bishop, and H. E. Varmus, manuscript in preparation), suggesting that the unintegrated DNA in transformed cells is synthesized by the viral polymerase from an RNA template. In this study, we used density labeling with 5-bromodeoxyuridine (BUdR) to document synthesis of the unintegrated DNA, showed the DNA to be present in the cytoplasm, and examined the mechanism of synthesis by analysis of the size of the strands and the pattern of density labeling.

MATERIALS AND METHODS

Cells and viruses. Propagation of Peking duck embryo cells and the B77 strain of ASV is described elsewhere (5-7). Cultures were infected at a multiplicity of infection of about 1 focus-forming unit/cell and passaged every 5 to 7 days by transferring cells from each 100-mm tissue culture plate to three plates.

Preparation of DNA. Cells were fractionated (5) and DNA was extracted from cytoplasmic and nuclear fractions as described in the accompanying manuscript (6) and elsewhere (7, 8).

Nucleic acid hybridization. B77 [^{32}P]complementary DNA (cDNA) (specific activity, ca. 5×10^7 to 10×10^7 counts/min per μg) and [^{32}P]70S RNA (specific activity, ca. 10×10^6 to 20×10^6 counts/min per μg) were prepared and annealed to gradient fractions as described in the accompanying paper (6). Annealing was assessed by resistance of cDNA to digestion by single-strand specific nuclease S1 or of 70S RNA to digestion by pancreatic ribonuclease and T1 ribonuclease; the quantity of viral DNA was determined from a calibration curve performed with increasing amounts of XC cell DNA (5, 6).

CsCl density gradients. A 6.0-ml amount of 0.01 M EDTA-0.01 M Tris-hydrochloride, pH 7.0, containing the sample of DNA was added to 8.2 g of CsCl (radio tracer grade; Harshaw Chemical Corp. Solon, Ohio, or Reliable Chemical Corp., St. Louis, Mo.). After dissolving the CsCl in a nitrocellulose tube, the tube was filled with mineral oil, capped, and centrifuged for 60 to 70 h at 33,500 rpm at 20 C in a Spinco type 40 rotor. The tube was punctured from below and 30- to 40-drop fractions were collected directly via a no. 20-gauge needle; a constant flow rate was obtained by coupling the top of the fluid column via a syringe to a Harvard pump.

Sucrose gradients. Samples were analyzed on 5 to 20% sucrose gradients in 0.1 M NaCl-0.01 M Tris-hydrochloride (pH 7.4)-0.001 M EDTA as described in the appropriate figure legends.

RESULTS

Viral DNA is newly synthesized in the cytoplasm of transformed cells. In an accompanying study, we found unintegrated DNA in cultures of ASV-transformed duck cells after several weekly passages of the cells (6). In two experiments, about 20 and 50% of viral DNA was detected in low-molecular-weight regions of alkaline sucrose gradients of ASV-transformed duck cell DNA in a zonal rotor; this DNA had the genome length "minus" strand and short "plus" strand characteristic of the major species of viral DNA observed in the cytoplasm shortly after infection (4a, 6). In addition, by analysis of viral DNA included in networks (7) of high-molecular-weight DNA from several cultures of similar transformed cells, about 4 to 6 copies were found integrated into cell DNA, but a variable amount (1 to 15 copies per cell) was found to be unintegrated, as estimated from enrichment of the supernatant fraction of DNA that did not sediment with the networks (6; unpublished data). When cells were fractionated into nuclear and cytoplasmic components, multiple copies of viral DNA with long "minus" and short "plus" strands were found in the cytoplasm; in contrast, over 80% of viral DNA in the nucleus was included in the high-molecular-weight DNA pellet formed by the procedure described by Hirt (2) (data not shown). These preliminary experiments, therefore, suggested that most or all of the unintegrated DNA was cytoplasmic (see below).

It seemed unlikely that the unintegrated viral DNA in these cells could be residual DNA formed during the acute infection several weeks earlier, since the cells had undergone at least 10 to 20 divisions in the interim. To document the cytoplasmic location of the unintegrated DNA and to demonstrate that it is newly synthesized in chronically infected cultures, we exploited our recent observation that viral DNA synthesized in acutely infected cells can be density labeled in both strands by addition of BUdR to the growth medium (4). ASV-transformed duck cells were exposed to BUdR (5 μg/ml) for 24 h, and the density of viral DNA in cytoplasmic (Fig. 1) and nuclear (Fig. 2) fractions was determined by centrifugation to equilibrium in cesium chloride gradients. At the time of density labeling, these cells had been passaged five times at weekly intervals subsequent to infection at a multiplicity of about 1 focus-forming unit/cell. Hybridization of samples to labeled cDNA and 70S RNA prior to density centrifugation measured 6 to 7 copies of viral DNA per cell in the nuclear fraction and about 10 copies per cell in the cytoplasmic fraction. Analysis of the density gradient of cyto-

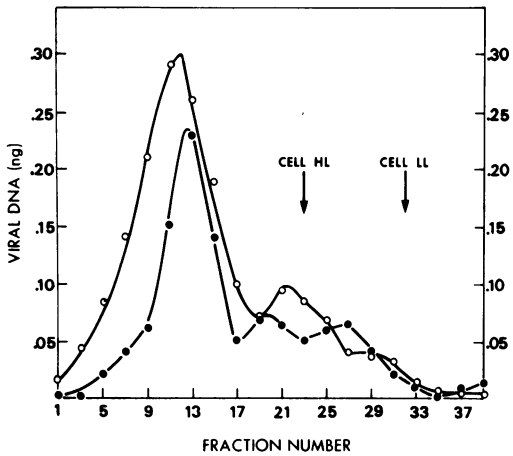

FIG. 1. *Density labeling of viral DNA in the cytoplasm of transformed duck cells. Duck embryo fibroblasts were infected with B77 ASV (multiplicity of infection about 1), passaged five times at 5- to 7-day intervals, labeled for 72 h with 0.05 μCi of [³H]thymidine per ml, and then incubated for 24 h with 5 μg of BUdR per ml. After fractionation of about 4 × 10⁸ cells, DNA was extracted from the cytoplasm (see text) and subjected to equilibrium centrifugation in a CsCl gradient in the Spinco type 40 rotor. Portions of fractions were annealed to either [³²P]70S RNA (○) or [³²P]cDNA (●) to measure "minus" or "plus" strands of viral DNA. Results are plotted as nanograms of viral DNA in 4% of each fraction, as determined from a calibration curve with XC cell DNA (see text). Density markers are provided by nuclear [³H]DNA contaminating the cytoplasm and banding in the positions of light (LL DNA, 1.700 g/cm³) or hybrid (HL DNA, 1.740 g/cm³), as indicated by the arrows.*

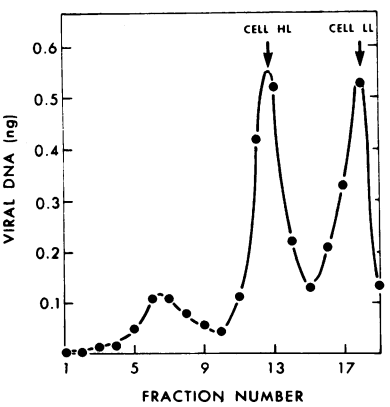

FIG. 2. *Viral DNA in the nucleus of BUdR-labeled, ASV-transformed duck cells. DNA was extracted from the nuclear fraction of cells studied in Fig. 1, sheared to a molecular weight of approximately 6 × 10⁶ by passage through a no. 26-gauge needle, and centrifuged to equilibrium in a CsCl density gradient in a type 40 rotor (100 μg of DNA, centrifuged for 60 h at 33,000 rpm at 20 C). Fractions were annealed with [³²P]cDNA; [³H]cell DNA served as density markers.*

plasmic DNA demonstrated that a large proportion (>80%) of viral DNA was labeled in both strands during the 24 h of incubation with BUdR (Fig. 1). Additional viral DNA was found in regions of intermediate density; as suggested below (see Fig. 4 and 5), this material was probably synthesized during the entry of BUdR into the nucleotide pool. Relatively small amounts of viral DNA were present in regions of the gradient in which DNA not labeled with BUdR should band; this could mean that very little viral DNA was synthesized prior to the labeling period or that unintegrated viral DNA was rapidly degraded in these cells or exported from the cells to the culture medium. As illustrated below (see Fig. 6), a relatively large proportion of unlabeled viral DNA is found in some cultures after incubation with BUdR for 24 h.

In contrast to the density of viral DNA in the cytoplasmic fraction, viral DNA in the nucleus comigrates principally with unreplicated (unlabeled) and once-replicated (half-labeled) cellular DNA. About half the viral DNA is in each region; a similar distribution of cellular DNA was determined from the distribution of [^{3}H]thymidine in this gradient, after correction for the reduced specific activity of hybrid DNA. (Since the period of [^{3}H]thymidine labeling precedes the addition of BUdR and since BUdR [at high concentration] prohibits further incorporation of [^{3}H]thymidine [at low concentration] into replicated DNA, hybrid DNA will have a specific activity approximately one-half that of light DNA.) The small amount of viral DNA fully labeled with BUdR in the nuclear fraction may reflect contamination of the nuclei with cytoplasmic material or a small amount of newly synthesized unintegrated viral DNA in the nucleus. It is unlikely that appreciable amounts of integrated viral DNA banded in the position of high-density DNA, since only about half the cells proceeded through a single S phase; a second S phase would be required to label integrated DNA in both strands.

Newly synthesized viral DNA has long "minus" strands and short "plus" strands. We have noted in the accompanying report that unintegrated viral DNA in transformed duck cells is composed of genome length "minus" strands and segmented "plus" strands (6). We observe this pattern in viral DNA synthesized after infection by ASV (4a, 6; Guntaka et al., manuscript in preparation) and consider it to be a "signature" of RNA-directed DNA synthesis by the viral polymerase. To test whether newly synthesized viral DNA in transformed cultures conformed to this pattern, we pooled viral DNA from regions of high and intermediate density in the gradient shown in Fig. 1; the DNA was thermally denatured, and the sedimentation of "plus" and "minus" strands in neutral sucrose gradients was monitored by annealing with [^{32}P]70S RNA and [^{32}P]cDNA (Fig. 3). Viral DNA from both density regions

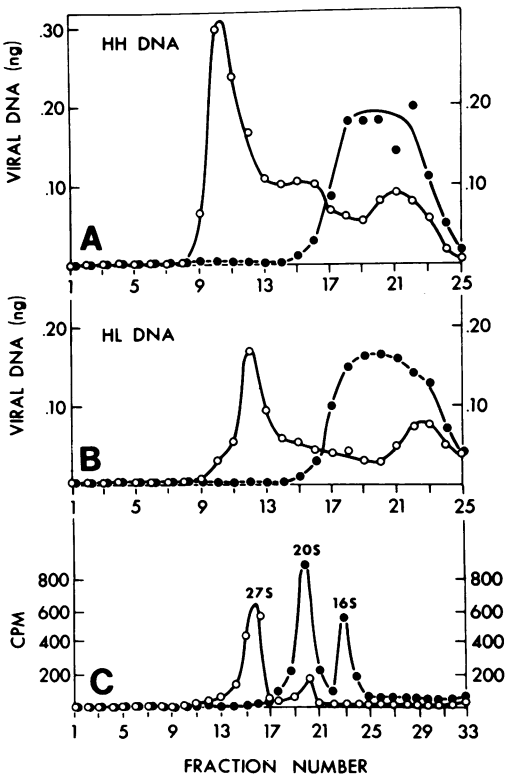

FIG. 3. *Size of strands of BUdR-labeled viral DNA in the cytoplasm of ASV-transformed duck cells. Viral DNA from the cytoplasm of ASV-transformed duck cells was pooled from fractions 5 to 17 (heavy density) and 20 to 27 (hybrid density) of the CsCl density gradient shown in Fig. 1. The pools were precipitated with ethanol, and portions were denatured (100 C for 5 min) and sedimented in a neutral 5 to 20% sucrose gradient for 15 h at 22,000 rpm (20 C) in a Spinco SW27.1 rotor. Approximately 20% of the heavy-density DNA (A) and 40% of the hybrid density DNA (B) were analyzed. The fractions were divided and annealed with [^{32}P]70S RNA (○) and [^{32}P]cDNA (●) to detect "minus" and "plus" strands of viral DNA. Simian virus 40 [^{32}P]DNA (forms I and II) (●) and pML21 [^{3}H]DNA (forms I and II) (○) were sedimented (without prior denaturation) in a parallel gradient (C). [^{3}H]cell DNA in the gradient of sedimented material of hybrid density (B) principally in 30–40S and 0–4S regions of the gradient, as determined by direct liquid scintillation counting. The simian virus 40 DNA was kindly provided by N. Mantei; pML21 is a supercoiled recombinant plasmid between mini-ColEl and the kanamycin resistance EcoRI fragment of pSC105 with a molecular weight of 6.7 × 10^6 (H. W. Boyer, personal communication).*

displayed very similar patterns: most of the "minus" strands sedimented at about 27–30S, consistent with linear single strands of about 2.5×10^6 to 3.0×10^6 daltons, although some shorter strands were also observed; the "plus" strands migrated at 6–10S, indicative of linear strands of 0.5×10^6 to 1.0×10^6 daltons. Native DNA from the heavy-density region banded broadly between 14S and 22S in a neutral sucrose gradient, as estimated by annealing with [^{32}P]cDNA (data not shown). These results suggest that most of the newly synthesized, unintegrated viral DNA exists in unusual duplexes of genome length "minus" strands and segmented "plus" strands; we consider this pattern to be a sign of DNA synthesis from an RNA template by the ASV polymerase in vivo. The absence of full-length "plus" strands indicates the absence of the covalently closed circular form of viral DNA observed in acutely infected duck cells (1). However, we have not excluded the possibility that the "minus" strands are in a circular form. In addition, it is likely that some of the viral molecules have appreciable regions of "minus" strand that are not in duplex with "plus" strands; this is suggested by the measurement of about 1.5 times more "minus" than "plus" strand in these gradients (Fig. 1 and 3) and by the asymmetry of annealing with 70S RNA and cDNA as shown in the major peak in Fig. 1. (DNA with single-stranded regions of "minus" strand would band at densities 0.010 to 0.015 g/ml higher than fully duplex DNA and would anneal more 70S RNA than cDNA.) It is worthy of note that the ^{3}H-labeled cell DNA from the region of intermediate density sedimented both faster and slower than the observed viral species, with very little ^{3}H-labeled DNA cosedimenting with viral species (see legend to Fig. 3B); this argues further for the nonintegrated status of the viral DNA.

Viral DNA of intermediate density is not replicated from a DNA template. The finding of viral DNA of intermediate density in the cytoplasm of transformed duck cells labeled with BUdR (Fig. 1; see also Fig. 6) raised the possibility that some or all viral DNA was replicated from a DNA template, yielding heavy-light hybrid DNA, rather than synthesized from an RNA template, yielding DNA labeled in both strands with BUdR. Although the sizing of strands of viral DNA (Fig. 3) suggested that it was made by the viral DNA polymerase from an RNA template, we further examined the viral DNA of intermediate density from the gradient shown in Fig. 1 to determine the pattern of labeling with BUdR. If such DNA were synthesized by replication from a DNA template, denaturation and rebanding in CsCl should reveal "plus" and "minus" strands banding at both heavy and light densities (Fig. 4A). If the DNA of intermediate density arises by reverse transcription during a period in which BUdR is entering the nucleotide pool, one of two patterns is likely: either "minus" strand will be of light density and "plus" strand will be of heavy density, if the strands are synthesized sequentially (Fig. 4B), or "minus" strand will be of intermediate density and the short "plus" strands will be of highly varied densities, if "plus" strand synthesis begins before completion of the "minus" strand (Fig. 4C). As a preliminary step, we showed (Fig. 5A) that viral DNA of hybrid density reequilibrated in a region of intermediate density when rebanded in a CsCl density gradient. After heat denaturation, the viral DNA was found to contain "minus" strands of intermediate density and "plus"

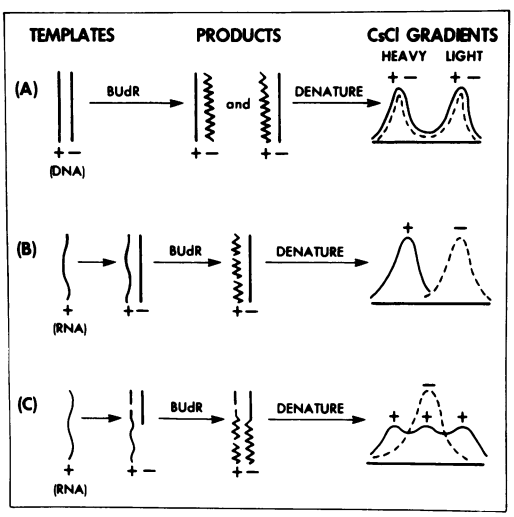

FIG. 4. *Predicted behavior of "hybrid" viral DNA after denaturation and rebanding in CsCl gradients, depending upon the mechanism of synthesis. (A) If viral DNA is replicated from a DNA template, hybrid DNA should be composed of heavy and light strands of both "plus" and "minus" polarities. (B) If viral DNA is synthesized from an RNA template during the entry of BUdR into the nucleotide pool, with sequential synthesis of "minus" and "plus" strands of DNA in the absence and presence of BUdR, the "minus" strand will band in the light region and the "plus" strand will band in the heavy region of a CsCl gradient. (C) If viral DNA is synthesized from an RNA template during the entry of BUdR into the nucleotide pool but "plus" strand synthesis is initiated before the completion of "minus" strand, hybrid DNA will be composed of a "minus" strand of intermediate density and "plus" strands of varied densities. Symbols: —, DNA strands; ≈, DNA strands labeled with BUdR; ~, RNA strands.*

strands of heavy, light, and intermediate densities (Fig. 5B). This result argues strongly against the synthesis of unintegrated viral DNA by DNA:DNA replication, favors the notion that the viral DNA is synthesized by reverse transcription, and suggests that the synthesis of "plus" strands commences before the completion of the "minus" strand. (Further data in support of this suggestion will be published elsewhere [R. V. Guntaka, O. C. Richards, P. R. Shank, J. M. Bishop, and H. E. Varmus, manuscript in preparation].)

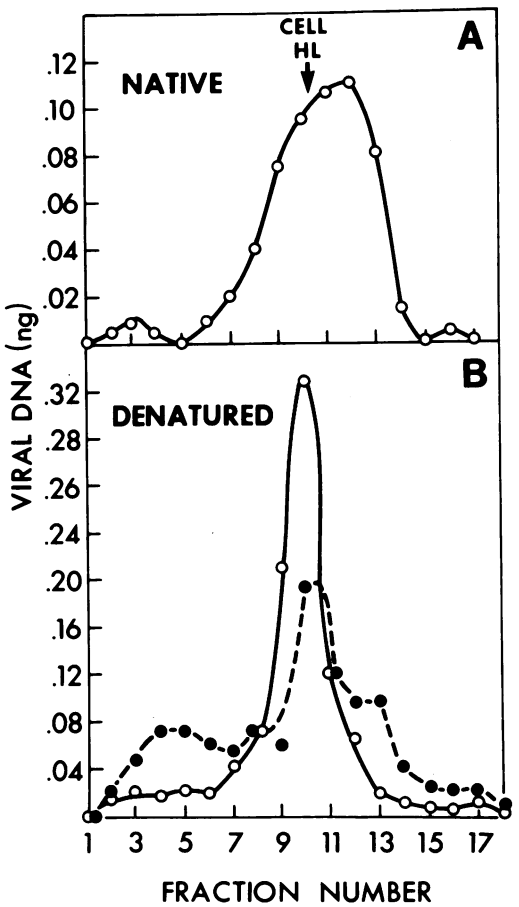

FIG. 5. *Analysis of viral DNA partially labeled with BUdR in CsCl density gradients. The pool of viral DNA of hybrid density from the cytoplasm of ASV-transformed duck cells (see Fig. 1 and 3) was centrifuged to equilibrium in CsCl density gradients (type 40 rotor, 20 C, 33,000 rpm, 60 h) in the native state ([A], 20% of pool) or after heat denaturation ([B], 40% of pool). Fractions from the gradient containing native DNA were annealed to [^{32}P]70S RNA (○); fractions from the gradient containing denatured DNA were annealed to both [^{32}P]70S RNA (○) and [^{32}P]cDNA (●).*

Chronically infected cultures are resistant to superinfection by B77 virus. Synthesis of unintegrated viral DNA in the cytoplasm of chronically infected duck cells could be caused by (i) reverse transcription of viral RNA in nascent virus particles in the virus-producing cells, (ii) fusion of infectious virus into infected cells in these crowded cultures, (iii) infection of previously uninfected cells, or (iv) superinfection of infected cells. We have asked whether infection or superinfection is possible in our chronically infected cultures by exposing the cells to BUdR for 24 h with and without 10 focus-forming units of B77 virus per cell. Exposure of uninfected cells to this quantity of virus leads to the synthesis of 3 to 10 copies of viral DNA per cell in 24 h (1, 4, 5); in this experiment, synthesis of as little as an additional 0.3 to 0.5 copy per cell should have been detectable. However, similar amounts of unintegrated viral DNA (about a 0.2 to 0.3 copy per cell) were synthesized during the labeling period in the control cells and in the cells we attempted to superinfect (Fig. 6A). Approximately 0.5 to 1.0 copy of viral DNA was present in the Hirt supernatant fractions from these cells, and the majority of the DNA banded at a density of about 1.71 g/cm^3, suggesting it was synthesized prior to the addition of BUdR. (Although a small quantity of viral DNA in this gradient may be derived from the 3% of nuclear DNA that did not enter the Hirt pellet, the density difference between viral and ^{3}H-labeled cell DNA suggests that most of the "light" viral DNA is unintegrated.) This experiment indicates that the majority of cells in these cultures was infected and not susceptible to superinfection.

It is evident from Fig. 1 and 6A that vastly different amounts of unintegrated viral DNA are found in different cultures of chronically infected cells. Another example is shown in Fig. 6B. Again, cells were infected with B77 virus, passaged six times at weekly intervals, and labeled with BUdR for 24 h. The cytoplasm of these cells contains about 1.5 copies of unintegrated DNA, most of which was synthesized prior to density labeling.

DISCUSSION

We have accumulated the following evidence to support our claim that unintegrated viral DNA is synthesized in the cytoplasm of ASV-transformed duck cells by viral DNA polymerase. (i) Variable amounts of unintegrated DNA (1 to 15 copies per cell) are detectable in these cells by sedimentation in alkaline sucrose gradients (6), by the network test (6), by Hirt fractionation (Fig. 6A), and by cell fractiona-

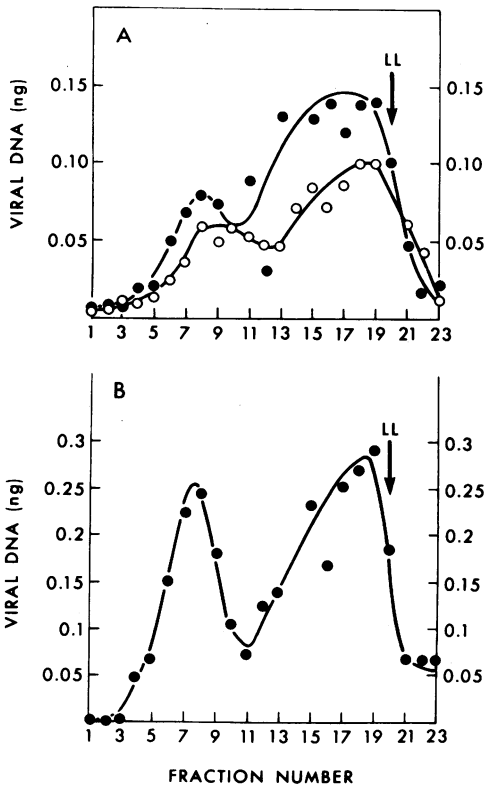

FIG. 6. *Unintegrated DNA in chronically infected cultures of duck cells after labeling with BUdR. (A) Duck embryo fibroblasts were infected with the B77 strain of ASV (multiplicity of infection, 1), passaged four times at weekly intervals, labeled for 12 h with [^{3}H]thymidine (0.2 μCi/ml), and incubated with BUdR (5 μg/ml) for 24 h. One set of cultures (1.8 × 10^8 cells) was superinfected with B77 virus (multiplicity of infection, about 10) at the time of addition of BUdR (○); a parallel set of cultures (1.4 × 10^8 cells) was not superinfected (●). The cells were fractionated by the Hirt procedure, and DNA was extracted from the supernatant fraction and centrifuged to equilibrium in CsCl density gradients. Fractions were annealed to [^{32}P]cDNA to determine the amount of viral DNA in each fraction. The arrow indicates the position of [^{3}H]cell DNA (light density) in these gradients. In these cultures, about 0.5 copy of viral DNA per cell was present in the Hirt supernatant, as determined from analysis prior to fractionation or from the annealing to gradient fractions. (B) Duck embryo fibroblasts were infected with B77 virus, passaged six times, labeled for 48 h with [^{3}H]thymidine (0.1 μCi/ml), and subsequently exposed to BUdR (5 μg/ml) for 24 h. DNA was extracted from the cytoplasm of 2 × 10^8 cells and centrifuged to equilibrium in a CsCl density gradient. Samples (5%) of each fraction were annealed with [^{32}P]cDNA (●) to determine the amount of viral DNA in each fraction. From this analysis, we estimate 1.5 copies of viral DNA to be present in the cytoplasm of each cell.*

tion (Fig. 1 and 6B). (ii) Cytoplasmic viral DNA can be labeled in both strands with BUdR (Fig. 1 and 6B) under conditions where less than 50% of the cells have passed through S phase. (iii) The pattern of labeling suggests transcription of DNA from an RNA template (Fig. 4 and 5). (iv) The unintegrated DNA consists of genome length "minus" strands and segmented "plus" strands, as found in viral DNA synthesized during acute infection by the viral polymerase (Fig. 3; reference 6; Varmus et al., in press; Guntaka et al., manuscript in preparation).

We have tested a large number of cultures of B77-transformed duck cells for this phenomenon, and we have observed considerable variation in the quantity of unintegrated DNA in these cultures (cf. Fig. 1 and 6). The large amount synthesized during the BUdR labeling period in the experiment shown in Fig. 1 through 5 was unusual and facilitated extensive analysis of the DNA products. More characteristic patterns are presented in Fig. 6. We have not been able to unambiguously identify factors that affect the rate of synthesis of unintegrated DNA, but sparse cells are less active than densely crowded cells (unpublished data). This may mean that synthesis is dependent upon fusion of virus budding from one cell into adjacent cells, but we cannot exclude the possibility that viral DNA is synthesized by nascent particles in virus-producing cells. On the other hand, we consider unlikely the possibility that we are observing DNA made during the secondary infection of cells that have remained uninfected in these cultures: the cells have been grown for several weeks following an initial high multiplicity of infection; they produce 10^6 to 10^7 focus-forming units of ASV per ml every 24 h; and they contain the four to eight copies of integrated viral DNA per cell that we generally find in chronically infected cultures. This issue can be unambiguously resolved only by testing clones of transformed cells, but we have been unable to propagate clones of B77-transformed duck cells selected in soft agar. However, our inability to augment synthesis of unintegrated viral DNA in chronically infected cultures by exposure to high multiplicities of B77 virus (Fig. 6A) suggests that a conventional infectious process is not responsible for the unintegrated DNA and that viral interference was established in these cultures.

We have attempted to exploit available mutants of ASV to examine further the mechanism of synthesis of unintegrated viral DNA in duck cells. However, we found a fourfold decrease in unintegrated DNA in cells chronically infected with wild-type B77 within 48 h after

shift from 35 C to 41 to 42 C. This phenomenon diminished the significance of the 10- to 20-fold decrease in unintegrated DNA repeatedly observed after temperature shift in cells infected by a viral mutant with a temperature-sensitive DNA polymerase (3, 4; unpublished data of E. Hunter and us). It is possible that transformed duck cells have limited tolerance for elevated temperatures, and we are investigating the possibility of repeating these experiments at slightly lower temperatures.

We do not know whether the phenomenon we have described here is peculiar to ASV-infected duck cells. We are not aware of reports of unintegrated viral DNA in chicken cells chronically infected with ASV or in cells chronically infected by mammalian RNA tumor viruses. The several ASV-infected mammalian cells we have examined likewise appear to lack unintegrated DNA after prolonged passage (4, 6, 7). Interestingly, in certain quail tumor cells infected by B77 ASV, we have found unintegrated DNA that persists as supercoiled duplexes in the nucleus; analysis of this species will be the subject of a separate report (R. V. Guntaka, O. C. Richards, P. R. Shank, H. J.Kung, N. Davidson, J. M. Bishop, and H. E. Varmus, manuscript in preparation).

It is doubtful whether the phenomenon we have described here has any significant role in the life cycle of the virus. We have presented evidence elsewhere (1) which argues that integration of viral DNA is required for subsequent events in virus replication. Since the cytoplasmic DNA appearing in these cultures does not appear to add to the integrated copies acquired in the initial phases of infection (e.g., in the first week), we do not see any necessity for its synthesis. However, the presence of such DNA may have important effects upon the interpretation of experiments that test the infectivity of DNA or the nature of integration sites. Moreover, under certain conditions, this species of viral DNA may be amenable to purification.

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