JOURNAL OF VIROLOGY, Nov. 1977, p. 461-469
Vol. 24, No. 2

Printed in U.S.A.

Inhibition of Viral DNA Synthesis in Stationary Chicken Embryo Fibroblasts Infected with Avian Retroviruses

EDWARD F. FRITSCH† AND HOWARD M. TEMIN*

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 23 May 1977

Previously, we reported (Fritsch and Temin, J. Virol. 21:119-130, 1977) that infectious viral DNA was not present in spleen necrosis virus-infected stationary chicken cells. However, a stable intermediate was present in such infected stationary cells as evidenced by the appearance of infectious viral DNA shortly after serum stimulation of these cells. After serum stimulation of infected stationary cells, the infectious viral DNA appeared first in the nucleus. In contrast, in infected dividing cells the infectious viral DNA appeared first in the cytoplasm. Significantly reduced amounts of complete plus- or minus-strand viral DNAs were detected by nucleic acid hybridization in stationary chicken cells infected with spleen necrosis virus or Schmidt-Ruppin Rous sarcoma virus compared with the amounts detected in infected dividing cells. These experiments indicated that infected stationary cells did not contain complete noninfectious copies of viral DNA. Furthermore, 5-bromodeoxyuridine labeling and cesium chloride density gradient centrifugation analysis of the infectious viral DNA that appeared after serum stimulation of infected stationary cells indicated that most viral DNA synthesis occurred after addition of fresh serum.

Production of progeny virus by cells infected with retroviruses requires that the cells pass through one normal replicative cell cycle after infection (5-7, 15). Cells infected in the nonreplicative phase of the cell cycle (stationary cells) do not synthesize viral RNA, viral proteins, or infectious virus until some time after a replicative cell cycle is initiated. Previous experiments involving inhibitors of DNA synthesis (16, 17) or 5-bromodeoxyuridine (BUdR) substitution into DNA and subsequent light irradiation (1, 2) have indicated, however, that some viral DNA is synthesized in infected stationary cells.

We have reported that infectious viral DNA is not present in spleen necrosis virus-infected stationary chicken cells (4). After addition of fresh serum and initiation of a normal replicative cell cycle, infectious viral DNA can be detected within 24 h, even if the addition of fresh serum is delayed for 6 days after infection (4). We now report that the amounts of complete plus- and minus-strand viral DNAs are significantly reduced in stationary chicken cells infected with either of two species of avian retroviruses compared with the amounts in infected dividing cells and that most viral DNA synthesis occurs after addition of fresh serum.

† Present address: Department of Microbiology, School of Medicine, University of Southern California, Los Angeles, CA 90033.

MATERIALS AND METHODS

Cells and viruses. Stationary cultures of chicken embryo fibroblasts were prepared by arresting cells in the non-replicative phase of the cell cycle as follows (4). Cultures of tertiary or later chicken embryo fibroblasts were prepared at 2×10^6 cells per 100-mm petri dish in 10 ml of Eagle minimal essential medium containing 20% tryptose phosphate broth (ET medium). After at least 4 h, the medium was changed to 10 ml of ET medium containing 1.5% fetal bovine serum, and the cultures were incubated at 37°C to deplete the serum of multiplication-stimulating activity. Four days after the addition of serum, 5 ml of Eagle medium without serum was added, and the cells were used 3 or 4 days later (stationary cells). All infections were carried out with such stationary cells. After infection, the culture medium was replaced with either the original depleted medium (these cells are called infected stationary cells) or medium containing fresh serum (these cells are called infected dividing cells).

In some experiments cells were labeled with 5 μg of BUdR (Calbiochem, Los Angeles, Calif.) per ml in Eagle medium with no serum or with serum dialyzed to remove any nucleosides. All manipulations with BUdR-labeled cells or DNA were carried out in darkened rooms, using a General Electric yellow lamp as the only light source.

Spleen necrosis virus (SNV) has been previously described (17). The Schmidt-Ruppin strain of Rous sarcoma virus, subgroup D (SR-RSV-D), was from a recently cloned stock and contained no detectable excess of transformation-defective virus. Hirt fractionation and cytoplasmic and nuclear fractionation of infected cells, DNA extraction, cesium chloride equi-

librium density gradient centrifugation, and assay of infectious viral DNA were as described previously (4). The amount of infectious viral DNA in a DNA sample was quantitated by end-point dilution as before (4) in terms of 50% infective dose (ID_{50}) units. Quadruplicate cultures of chicken embryo fibroblasts were exposed to two- or threefold serial dilutions of DNA, and the amount of DNA necessary to infect 50% of the recipient cultures (one ID_{50} unit) was calculated (11).

Nucleic acid hybridization. A ^{3}H-labeled complementary DNA (cDNA) probe was prepared from disrupted virions of SNV as described previously (4). The [^{3}H]cDNA had a specific activity of approximately 2×10^7 dpm/μg and hybridized 40% to 20 μg of total DNA from chicken cells chronically infected (18) with SNV (data not shown). [^{3}H]uridine-labeled viral RNA was prepared by labeling SNV- or Rous sarcoma virus–Rous-associated virus-O (RSV-RAV-O)-infected chicken or pheasant cells in 100-mm plastic petri dishes with 4 ml of Eagle medium containing 100 μCi of [^{3}H]uridine (New England Nuclear Corp., Boston, Mass.; specific activity, 49 Ci/mmol) per ml and dialyzed serum (3% calf serum and 3% fetal calf serum). Cells were prelabeled with the [^{3}H]uridine for 12 h; then fresh radioactive medium was applied, and the cells were harvested at three 12-h intervals. ^{3}H-labeled 60–70S viral RNA was isolated by published procedures (8). The SNV [^{3}H]RNA hybridized 82% to 100 μg of total DNA from chicken cells chronically infected with SNV (data not shown). RSV-RAV-O [^{3}H]RNA was a kind gift of E. Keshet. The [^{3}H]cDNA and the [^{3}H]RNA probes detect the plus- and minus-strand viral DNAs, respectively (using the convention that the DNA strand identical in polarity to the viral mRNA is designated the plus strand).

Unlabeled DNA for nucleic acid hybridization analysis was fragmented by alkaline hydrolysis (0.3 M NaOH, 100°C, 20 min), neutralized, and ethanol precipitated as described (4). Nucleic acid hybridization with cDNA or 60–70S viral RNA was in 0.6 M NaCl, 0.1% sodium dodecyl sulfate, 1% phenol, 0.001 M EDTA, and 0.05 M Tris (pH 7.3) for 60 to 70 h at 63°C as described previously (4). The percentage of hybridization was determined after digestion with S1 nuclease (4) or RNase A (8).

RESULTS

Appearance of infectious viral DNA after addition of fresh serum to infected stationary cells. We previously showed that infectious viral DNA was not present in stationary chicken embryo fibroblasts infected with SNV (4). No infectious viral DNA was found for up to 5 days after infection if cells were maintained in the absence of fresh serum. However, infectious viral DNA did appear within 24 h after addition of fresh serum to these cells.

For example, 48 h after infection of stationary chicken embryo fibroblasts with SNV, there was more than a 10-fold difference in the amounts of infectious viral DNA recovered from cells that did and did not receive fresh serum at the time of infection (Fig. 1A, 0 h after serum). When fresh serum was added to infected stationary cells that were maintained in the absence of fresh serum for 48 h after infection, there was a sharp increase 12 to 18 h later in the amounts of infectious viral DNA recovered in both the Hirt supernatant and pellet fractions. An additional increase occurred by 48 h after addition of fresh serum.

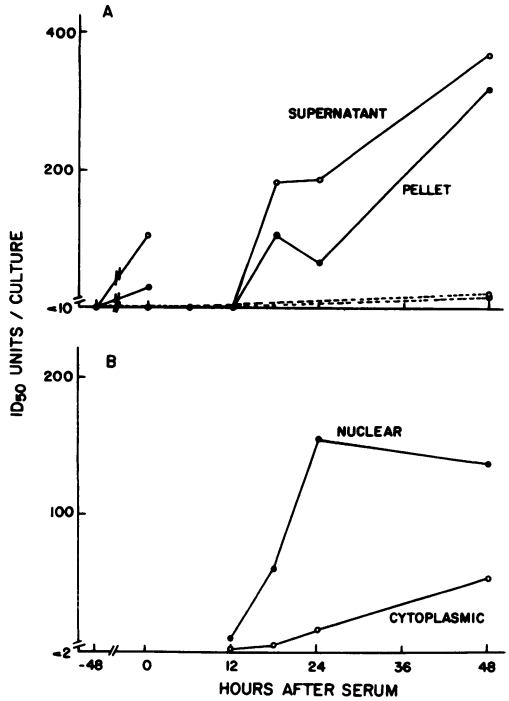

FIG. 1. *Kinetics of appearance and cellular location of infectious SNV DNA after addition of fresh serum to infected stationary cells. Cultures of stationary chicken embryo fibroblasts (approximately 4 × 10^6 cells per culture) were infected with SNV at a multiplicity of approximately 10 PFU/cell, and medium with fresh serum (solid line) or the original depleted medium without fresh serum (dashed line) was replaced. Forty-eight hours after infection (0 h after serum), the medium on some of the cultures was replaced with medium containing fresh serum, and at different times after addition of fresh serum three cultures were harvested by Hirt fractionation (A) or ten cultures were harvested for cytoplasmic and nuclear fractionation (B). Each sample was assayed for infectious viral DNA. The recovery of infectious viral DNA in the combined cellular fractions was for unexplained reasons two- to fourfold lower than in the combined Hirt fractions. However, this reduced recovery was not observed in other experiments that had an otherwise similar result. Symbols: ○, Hirt supernatant infectious viral DNA (A) or cytoplasmic infectious viral DNA (B); ●, Hirt pellet infectious viral DNA (A) or nuclear infectious viral DNA (B).*

The distribution of infectious viral DNA between the cytoplasmic and nuclear fractions of the same cells at 12, 18, 24, and 48 h after

addition of fresh serum is presented in Fig. 1B. At 18 or 24 h after addition of fresh serum, approximately 90% of the infectious viral DNA was present in the nuclear fraction. Only by 48 h after addition of fresh serum did the cytoplasmic fraction contain a significant proportion of the infectious viral DNA.

We previously showed (4) that at 1 and 2 days after infection of dividing cells, approximately 50% of the infectious viral DNA was in the cytoplasmic fraction. The reproducible differences observed in the proportions of infectious viral DNA in the nuclear fractions of infected dividing cells and of infected stationary cells after addition of fresh serum indicate that the site of appearance of infectious viral DNA is distinctly different in these cells.

These experiments demonstrate that a stable viral intermediate is present in infected stationary cells. Subsequent experiments attempted first to determine whether this intermediate consisted of complete noninfectious viral DNA.

Reduced amounts of complete viral DNA in stationary cells. Synthesis of viral DNA is apparently blocked in infected stationary cells at a step before the formation of the infectious linear double-stranded viral DNA (4; Fig. 1). Since infectious unintegrated viral DNA can be isolated from the supernatant fraction of a Hirt fractionation (4), it seemed likely that the intermediate present in infected stationary cells would also be in the Hirt supernatant fraction. Therefore, we attempted to detect this intermediate in the Hirt supernatant fraction of infected stationary cells by using nucleic acid hybridization with probes that detect either the plus- or the minus-strand viral DNA.

DNA was prepared from the Hirt supernatant fractions of stationary or dividing chicken embryo fibroblasts 48 h after infection with SNV. At this time, there is in infected dividing cells a peak in the amount of unintegrated infectious viral DNA (4). Each DNA preparation was then hybridized to SNV [^{3}H]cDNA or [^{3}H]RNA to determine the amounts of plus- and minus-strand viral DNAs present and titered to determine the amount of infectious viral DNA.

There were significantly less hybridizable plus- and minus-strand viral DNAs in the DNA preparation from infected stationary cells compared with that from infected dividing cells (Fig. 2). These differences can be quantitated by comparing the amounts of DNA required to protect a given proportion of the ^{3}H-labeled probes. In each case, approximately 10-fold more DNA from infected stationary cells was required to protect 10% of the ^{3}H-labeled probes than DNA from infected dividing cells.

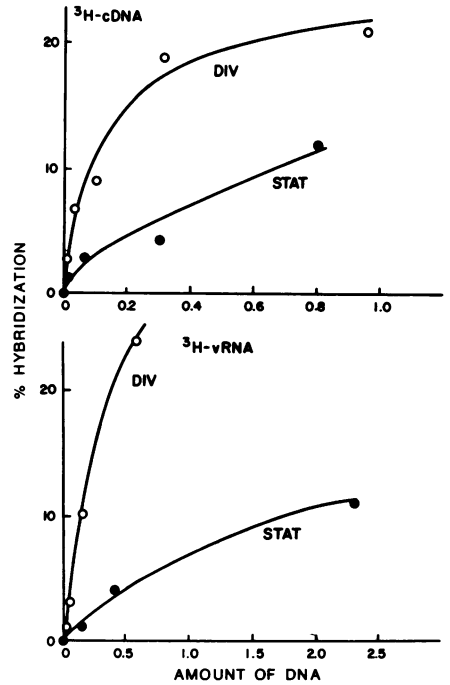

FIG. 2. *Nucleic acid hybridization of SNV [^{3}H]-cDNA or [^{3}H]RNA to Hirt supernatant DNA from SNV-infected stationary or dividing cells. [^{3}H]-cDNA prepared from disrupted virions of SNV or SNV [^{3}H]RNA (2,000 dpm each) was hybridized to different amounts of Hirt supernatant DNA prepared from parallel cultures of stationary (STAT, ●) or dividing (DIV, ○) cells 2 days after infection with SNV. The abscissa represents the amount of Hirt supernatant DNA in each hybridization reaction in terms of the number of cultures from which that DNA was prepared. Hybridization and S1 nuclease or RNase A digestion were as described in Materials and Methods. Each point represents the average of duplicate samples. The [^{3}H]cDNA probe had an intrinsic nuclease resistance of 13%, which was subtracted. The [^{3}H]RNA probe had an intrinsic nuclease resistance of 3%, which was subtracted.*

To insure that the intermediate was not present in the Hirt pellet DNA, we performed a similar hybridization experiment with Hirt pellet DNA from infected stationary cells. Reduced amounts of Hirt pellet infectious viral DNA are observed in infected stationary cells compared with infected dividing cells (4; Fig. 1; and data not shown). No significant hybridization of labeled viral RNA with DNA from the Hirt pellet fraction of infected stationary cells was detected (data not shown).

Similarly, SR-RSV-D-infected stationary cells contained significantly less hybridizable minus-strand viral DNA than did infected dividing cells (Fig. 3). SR-RSV-D-infected stationary chicken

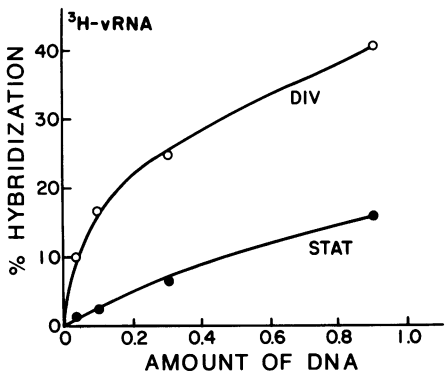

FIG. 3. *Nucleic acid hybridization of RSV-RAV-O [^{3}H]RNA to Hirt supernatant DNA from SR-RSV-D-infected stationary or dividing cells. RSV-RAV-O [^{3}H]RNA (1,000 dpm) was hybridized to different amounts of Hirt supernatant DNA prepared from parallel cultures of stationary (STAT, ●) or dividing (DIV, ○) cells 2 days after infection with SR-RSV-D. (RSV-RAV-O and SR-RSV-D RNAs have extensive nucleic acid sequence homology [8], and therefore RSV-RAV-O [^{3}H]RNA was used as a probe in these experiments because of availability.) Hybridization and RNase A digestion were as described in Materials and Methods. Each point represents the average of duplicate samples. The [^{3}H]RNA had an intrinsic nuclease resistance of 0.5%, which was not subtracted. Control experiments with Hirt supernatant DNA from uninfected cells showed no significant hybridization.*

cells also contained reduced amounts of infectious viral DNA (data not shown).

These experiments indicate that infected stationary cells do not contain amounts of complete noninfectious viral DNA similar to the amounts of complete infectious viral DNA in infected dividing cells.

The hybridizable viral DNA detected in the DNA preparations from infected stationary cells can be explained either by the presence of partial copies of the plus- and minus-strand viral DNAs or by the low background of infectious viral DNA in the infected stationary cells (approximately 10-fold less infectious viral DNA was recovered from infected stationary cells than from infected dividing cells [Fig. 1A and data not shown]). Per infectious viral DNA molecule, there were approximately the same amounts of hybridizable viral DNA in the Hirt supernatant fractions either from infected stationary or dividing cells or in purified linear viral DNA (Fig. 4). Furthermore, the hybridizable minus-strand viral DNA that was present in infected stationary cells sedimented in an alkaline sucrose gradient (Fig. 5) with the approximate rate expected for full-length single-stranded viral DNA (14). No small viral DNA from infected stationary cells was detected as slower-sedimenting material. These data, therefore, indicate that the hybridizable DNA detected in the DNA preparations from infected stationary cells is the result of the background of infectious viral DNA in these cells and not of the presence of partial copies of the viral DNA. This background of infectious viral DNA is consistent with viral DNA synthesis in the small proportion of cells that proceed through the replicative cell cycle in the absence of fresh serum (4, 5).

The appearance of hybridizable viral DNA after serum stimulation of infected stationary cells was also examined. In the experiment described in Fig. 1, we found that the appearance of minus-strand viral DNA in the Hirt supernatant fractions exactly paralleled the appearance of infectious viral DNA. That is, only a small

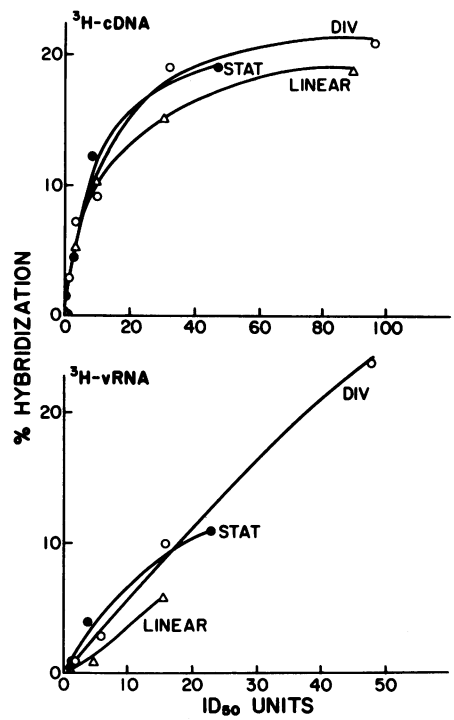

FIG. 4. *Nucleic acid hybridization to Hirt supernatant infectious viral DNA from SNV-infected stationary or dividing cells or to purified linear infectious viral DNA. The data from Fig. 2 are replotted after normalization to the number of ID_{50} units of each DNA per hybridization reaction. Also included is hybridization to purified linear viral DNA (LINEAR, △) prepared from infected dividing cells (4). As stated in the text and in reference 4, infected stationary cells contain a reduced, but detectable, amount of infectious viral DNA. This reduced level of infectivity was used in normalizing the hybridization results for the infected stationary cell DNA. Other symbols are as in Fig. 2.*

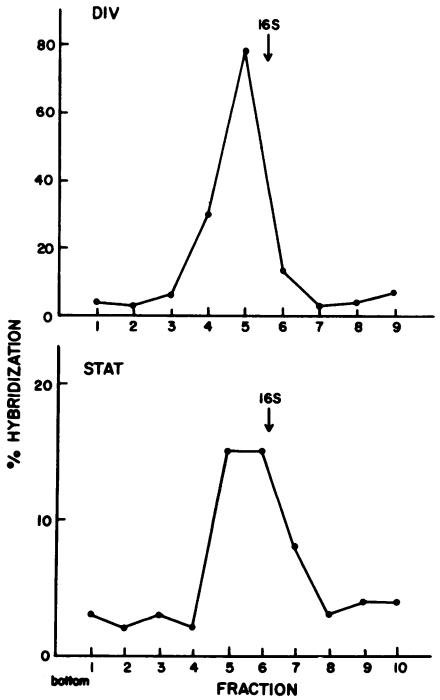

FIG. 5. *Sedimentation in alkaline sucrose gradients of Hirt supernatant DNA from SR-RSV-D-infected stationary or dividing cells. Hirt supernatant DNAs from the experiment of Fig. 3 representing six cultures each of stationary or dividing infected cells were centrifuged in parallel 10 to 30% alkaline sucrose gradients in 0.3 M NaOH-0.5 M NaCl-0.005 M EDTA for 5 h at 48,000 rpm in a Spinco SW50.1 rotor. Phage fd ^{3}H-labeled DNA (molecular weight, 1.7 × 10^6) was centrifuged in a parallel gradient as a sedimentation marker (13). After fractionation, each fraction was heated at 100°C for 20 min to fragment the DNA and hybridized to 1,000 dpm of RSV-RAV-O [^{3}H]RNA. The [^{3}H]RNA had an intrinsic nuclease resistance of 2%, which was subtracted.*

amount of minus-strand viral DNA was detected in cells without fresh serum; there was no increase in the amount of minus-strand viral DNA by 12 h after addition of fresh serum; and there was approximately a 12-fold increase 12 to 18 h after addition of fresh serum (data not shown).

These experiments indicate that infected stationary cells contain only small amounts of complete plus and minus strands of viral DNA, consistent with the small fraction of infected stationary cells that proceed through the replicative cell cycle in the absence of fresh serum. We next attempted to determine whether, in addition, synthesis of a portion of the viral DNA molecule occurs in most infected stationary cells.

Labeling with BUdR of viral DNA synthesized in infected stationary cells. Evidence that viral DNA is synthesized in most infected stationary cells has come from experiments in which BUdR was present in the medium after infection (1, 2). Subsequent sensitivity of the infection to light irradiation indicated that incorporation of the BUdR (and therefore viral DNA synthesis) did occur in most of the infected stationary cells. These experiments were performed with avian sarcoma viruses (Schmidt-Ruppin Rous sarcoma virus and the Bryan strain of Rous sarcoma virus). We have confirmed them with a reticuloendotheliosis virus, SNV (Table 1).

Since incorporation of BUdR into DNA leads to an increase in the buoyant density of DNA in CsCl equilibrium density gradients (9), it should be possible to determine what proportion of the viral genome is synthesized in infected stationary cells by labeling the DNA synthesized in infected stationary cells with BUdR and examining the density of infectious viral DNA appearing soon after addition of fresh serum in the absence of BUdR.

In an experiment done in parallel with the

TABLE 1. *Comparison of light inactivation of SR-RSV-D- and SNV-infected stationary chicken cells*[a]

Virus	BUdR	No. of cells plated/infectious center[b]
SR-RSV-D	−	10^4
SR-RSV-D	+	5×10^4
SNV	−	6×10^3
SNV	+	30×10^3

[a] Stationary chicken embryo fibroblasts were infected with either SR-RSV-D or SNV at a low multiplicity, and Eagle medium containing no or 5 μg of BUdR per ml was replaced. Twenty-four hours later, each culture was washed twice with 2 ml of a buffer containing 0.14 M NaCl, 0.005 M KCl, 0.0007 M Na_2HPO_4, 0.001 M $CaCl_2$, 0.0005 M $MgCl_2$, and 0.025 M Tris (pH 8.0), 6 ml of the same buffer was replaced, and the cultures were exposed for 1 h to light from two Westinghouse FS-20 fluorescent sun lamps from the underside at a 6-cm distance and through a filter consisting of a 300-μg/ml thymidine solution in water in a Falcon tissue culture flask (path length, 3 cm). This lamp emits a peak efficiency at 310 nm. After exposure, the cells were trypsin treated, washed twice, counted, and seeded at serial 10-fold dilutions (in quadruplicate) onto freshly prepared cultures of chicken embryo fibroblasts; medium with fresh serum was added, and the cultures were examined for the presence or absence of virus several days later. Control cultures incubated with or without BUdR and with or without light exposure demonstrated that virus production (SNV) or focus formation (SR-RSV-D) was inhibited in cultures exposed to BUdR and light and that there was no effect of light exposure or BUdR incorporation per se on either of the above parameters (unpublished data).

[b] Calculated number of cells required to register as an infectious center.

experiment described in Table 1, the medium of some SNV-infected cells was changed 48 h after infection from medium without serum and with BUdR to medium with fresh serum and without BUdR. Infectious viral DNA was prepared from the Hirt supernatant fraction of these cells 24 h after addition of fresh serum and was centrifuged to equilibrium in a CsCl density gradient (Fig. 6).

The infectious viral DNA in this CsCl gradient had a density slightly greater than the density of chicken cellular DNA not substituted with BUdR. This density is indistinguishable from the density of unintegrated infectious SNV DNA (4). Therefore, the infectious viral DNA that appeared after addition of fresh serum did not contain BUdR in a significant portion of the genome. BUdR incorporation into the equivalent of 20% of one strand of the viral DNA would have resulted in a detectable shift in the density of the infectious viral DNA.

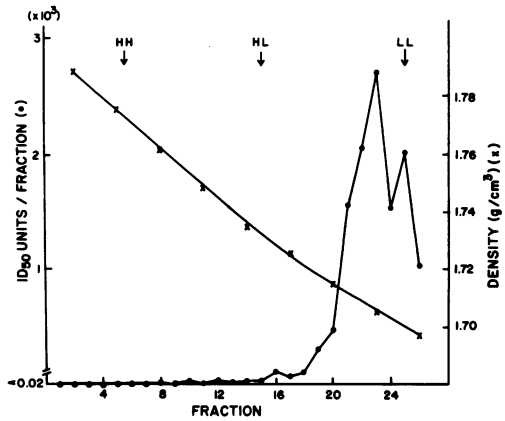

FIG. 6. *Cesium chloride equilibrium density gradient centrifugation of infectious SNV DNA after addition of fresh serum to infected stationary cells labeled for 48 h with BUdR in the absence of serum. Ten cultures of stationary chicken cells were prepared in parallel with the cells described in Table 1 and infected with SNV at a multiplicity of infection of about 10 PFU/cell, and Eagle medium without serum and containing 5 μg of BUdR per ml was replaced. After 48 h of incubation at 37°C, the medium was changed to Eagle medium with 3% calf serum and with 3% fetal calf serum and no BUdR, and the cells were harvested by Hirt fractionation 24 h later. There was approximately a 30-fold increase in the amount of infectious viral DNA per culture in the cells 24 h after serum stimulation. There was no detectable increase in virus production in the cells 24 h after serum stimulation (data not shown). The Hirt supernatant DNA was purified and then sedimented to equilibrium in a cesium chloride density gradient (starting density 1.735 g/cm³, 62 h, 18°C, 33,000 rpm, Spinco type 40 rotor). ³H-labeled marker DNAs, consisting of native chicken cellular DNA (LL) and chicken cellular DNAs substituted in one strand with BUdR (HL) or in both strands with BUdR (HH) (prepared from chicken cells labeled with 5 μg of BUdR per ml for 24 h and with [³H] cytidine and 5 μg of BUdR per ml for an additional 24 h), were added as internal density markers. The gradient was fractionated from the bottom, refractive indexes of selected fractions were determined, and a portion of each fraction was counted to determine the positions of the marker DNAs. Each fraction was dialyzed extensively against 0.015 M NaCl–0.15 M sodium citrate (pH 7.0) to remove the CsCl and then quantitatively assayed for infectious viral DNA.*

In similar experiments using SR-RSV-D, the infectious viral DNA had a density only slightly greater than the density of chicken cellular DNA not substituted with BUdR (Fig. 7). Therefore, the infectious SR-RSV-D DNA that appeared after fresh serum addition also did not contain BUdR in a significant portion of the genome.

In both of these experiments (Fig. 6 and 7), only very small amounts of infectious viral DNA were found in the denser regions of the gradients, and, in particular, no infectious viral DNA of hybrid density (which would band at a density slightly greater than that of the HL marker DNA) was observed.

The proportion of the viral genome synthesized in stationary cells was also determined by BUdR labeling of the DNA synthesized after addition of fresh serum to infected stationary cells. In such a reciprocal experiment in which

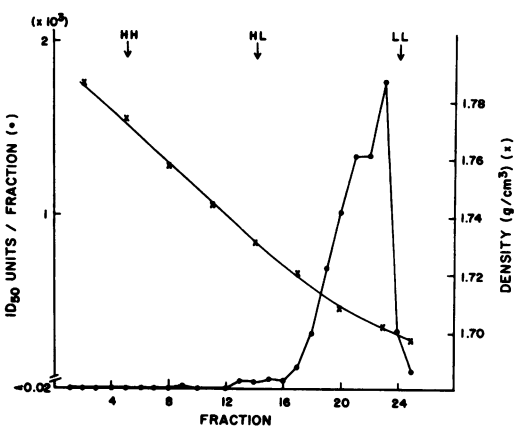

FIG. 7. *Cesium chloride equilibrium density gradient centrifugation of infectious SR-RSV-D DNA after addition of fresh serum to infected stationary cells labeled for 48 h with BUdR in the absence of serum. This experiment was performed as that described in Fig. 6 except the cells were infected with SR-RSV-D at a multiplicity of infection of about 5 focus-forming units/cell. There was approximately a 20-fold increase in the amount of infectious viral DNA per culture in the cells 24 h after serum stimulation. There was no detectable increase in virus production 24 h after serum stimulation (data not shown).*

BUdR was added at the time of addition of fresh serum to SNV-infected stationary cells, the majority of the infectious viral DNA banded in a CsCl gradient as two peaks near the density of DNA fully labeled with BUdR (Fig. 8). The peak of infectious viral DNA banding at a density slightly higher than that of the HH marker DNA is consistent with the occurrence of viral DNA fully labeled with BUdR. The peak of infectious viral DNA banding at a density slightly lower than that of the HH marker DNA is consistent with results obtained with viral DNA labeled with BUdR in all but the equivalent of 20 to 30% of one strand of the viral DNA. This latter result indicates that synthesis of 20 to 30% of one strand of the viral DNA occurs in some infected stationary cells.

The experiments presented in this paper, therefore, indicate that viral DNA synthesis is controlled by the replicative cell cycle. The major portion of viral DNA synthesis after infection of stationary cells only occurs after the cells are stimulated by fresh serum to enter the replicative cell cycle.

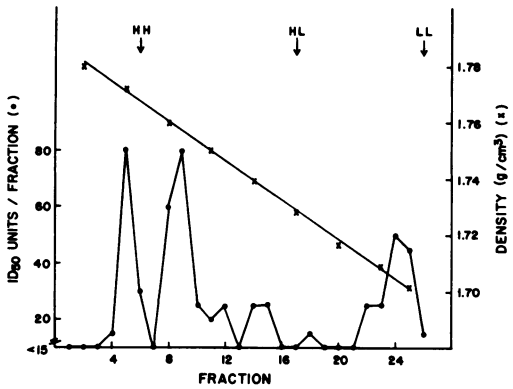

FIG. 8. *Cesium chloride equilibrium density gradient centrifugation of infectious SNV DNA from cells labeled with BUdR for 24 h after addition of fresh serum to infected stationary cells. Seven cultures of stationary chicken cells were prepared and infected with SNV at a multiplicity of infection of 10 PFU/cell, and the original depleted medium was replaced. After 48 h of incubation at 37°C, the medium was replaced with Eagle medium with 3% dialyzed calf serum and with 3% dialyzed fetal calf serum and 5 μg of BUdR per ml. The cells were harvested by Hirt fractionation 24 h later. There was approximately a fivefold increase in the amount of infectious viral DNA per culture in the cells 24 h after serum stimulation. There was no detectable increase in virus production in the cells 24 h after serum stimulation (data not shown). DNA from the Hirt supernatant fraction was then centrifuged and assayed as described in the legend to Fig. 6.*

DISCUSSION

Chicken cells infected by avian retroviruses do not synthesize progeny virus if they are prevented from entering a normal replicative cell cycle after infection. Treatments that interfere with the normal replicative cell cycle, including X-irradiation and serum starvation (5, 6, 12, 15), prevent the appearance of progeny virus.

Stationary chicken cells prepared by serum starvation do not synthesize viral RNA after infection until some time after a replicative cell cycle is initiated by addition of fresh serum (6). Addition of fresh serum results in the appearance of both viral RNA and progeny virus 18 to 24 h later, even if addition of fresh serum is delayed for several days (5, 6). Therefore, a normal replicative cell cycle is required early in the replication cycle of the virus before or at the initiation of transcription of viral RNA. Furthermore, the viral information must be present in infected stationary cells in a stable form.

We have attempted to determine at what stage the viral replication cycle is arrested in infected stationary cells by examining the synthesis of viral DNA in infected stationary cells. Our results indicate that both infectious viral DNA and hybridizable viral DNA are present in reduced amounts in infected stationary cells compared with the amounts in infected dividing cells and, therefore, that a normal replicative cell cycle is required for the synthesis of viral DNA. The requirement for a normal replicative cell cycle was observed with both the avian leukosis-sarcoma virus species and the reticuloendotheliosis virus species of avian retroviruses.

While this research was in progress, Varmus et al. similarly observed reduced amounts of complete plus- and minus-strand viral DNAs in stationary quail embryo fibroblasts infected with the B77 strain of avian sarcoma virus (19). However, they did detect, also, some plus- and minus-strand viral DNAs in infected stationary cells that were shorter in length than viral DNAs in infected dividing cells.

Infected stationary cells contain the viral genetic information in a stable form (Fig. 1; 4, 5), possibly as DNA, RNA, or a combination of DNA and RNA. The cellular location of this stable intermediate in infected stationary cells was first examined. In contrast to the cytoplasmic appearance of infectious viral DNA after infection of dividing cells (4), the infectious viral DNA that first appeared after addition of fresh serum to infected stationary cells was primarily in the nuclear fraction. This result indicates that the intermediate present in the infected stationary cells probably migrates to the nucleus or to a site near the nucleus.

The nature of the intermediate present in

infected stationary cells was examined by BUdR labeling and nucleic acid hybridization experiments. The inactivation of virus infection by light irradiation of stationary cells infected in the presence of BUdR (Table 1; 1, 2) and the effect of inhibitors of DNA synthesis on virus infection in stationary cells (15–17) indicate that some viral DNA synthesis does occur in infected stationary cells. However, the BUdR-labeling–buoyant density experiments (Fig. 6–8) indicate that the major portion of viral DNA synthesis occurs after addition of fresh serum. Also, the nucleic acid hybridization experiments (Fig. 2–4) indicate that complete plus- and minus-strand viral DNAs are not present in as many copies in infected stationary cells as in infected dividing cells.

Our results can be explained in two ways. First, only a small segment of the complete viral DNA molecule might be synthesized in all infected stationary cells. Synthesis of this segment of viral DNA in the presence of BUdR renders the infection sensitive to light irradiation (Table 1). After addition of fresh serum, the cells enter the replicative cell cycle, and the remaining portion of the genome is copied into DNA. The segment of viral DNA made in stationary cells is below or at the limit of detection by the nucleic acid hybridization or the BUdR-labeling–buoyant density experiments. (The limit of detection by the nucleic acid hybridization experiments is difficult to assess because of the background of infectious viral DNA in infected stationary cells. We estimate conservatively that synthesis of 25% of one strand of the viral DNA would have been detected. As stated in Results, the limit of detection by the BUdR-labeling–buoyant density experiments is synthesis of the equivalent of 20% of one strand.) This hypothesis explains the hybridizable and infectious viral DNA present in infected stationary cells as a background resulting from the small proportion of cells in the infected stationary cell population that enter the replicative cell cycle in the absence of fresh serum.

Alternatively, a limited number of complete viral DNA molecules might be synthesized in all infected stationary cells and later be amplified by rapid viral DNA replication after addition of fresh serum. The difference in the amounts of hybridizable and infectious viral DNA between infected stationary and infected dividing cells would be the result of amplification by replication only in infected dividing cells. The hybridizable and infectious viral DNA detected in infected stationary cells would be the result of synthesis of the complete viral DNA molecules from infectious virions without additional replication of the viral DNA.

However, some of the hybridizable and infectious viral DNA detected in infected stationary cells probably results from replication of viral DNA in infected stationary cells that enter the replicative cell cycle in the absence of fresh serum. The finding of small amounts of Hirt pellet infectious viral DNA in infected stationary cells is consistent with this explanation. Therefore, under the second hypothesis there must be considerable amplification of viral DNA after addition of fresh serum.

In addition, no hybrid density viral DNA (HL) was detected in the gradients of Fig. 6 and 7. HL viral DNA should represent a detectable portion of the replicated viral DNA in these experiments.

We cannot distinguish conclusively between these hypotheses. However, in the experiment of Fig. 8, a peak of infectious viral DNA was present at a density indicating incorporation of BUdR into the equivalent of 20 to 30% of one strand of viral DNA in some infected stationary cells. This finding favors the hypothesis that synthesis of a segment of viral DNA occurs in infected stationary cells and that completion of the viral DNA occurs after addition of fresh serum. We are presently attempting to test this hypothesis by determining whether a specific region(s) of the viral genome is copied into DNA in infected stationary cells.

The mechanism(s) by which the replicative cell cycle controls the synthesis of viral DNA is unknown. Specific cell factors, normally present in dividing cells but depleted in stationary cells, might be required at particular early stages in the synthesis of viral DNA. More general factors, such as availability of deoxyribonucleotide precursors either as a result of effects on the pool sizes (which appear to be only slightly reduced, if at all, in stationary chicken cells; see reference 3) or on their cellular localization, might also result in decreased viral DNA synthesis. In addition, mechanisms involving specific degradation of viral DNA in infected stationary cells, analogous to bacterial restriction (10), cannot be ruled out.

The amounts of both infectious viral DNA and minus-strand viral DNA increased 12 to 18 h after addition of fresh serum to infected stationary cells. Although not directly studied in this work, this period corresponds to mid to late S phase as reported previously (6). The synthesis of viral DNA after addition of fresh serum is likely due to the reappearance during S phase of the factor(s) necessary for completion or replication of the viral DNA.

ACKNOWLEDGMENTS

We thank Susan Hellenbrand and Neil Wilson for excellent

technical assistance; E. Keshet for materials; and N. Battula, L. Estis, E. Keshet, S. Mizutani, J. O'Rear, and S. Weller for helpful comments on the manuscript.

This investigtion was supported by Public Health Service Program Project Grant CA-07175 from the National Cancer Institute. E.F.F. was supported by Public Health Service Training Grant TO1-CA-5002 from the National Cancer Institute. H.M.T. is an American Cancer Society Research Professor.

LITERATURE CITED

1. **Balduzzi, P., and H. R. Morgan.** 1970. Mechanism of oncogenic transformation by Rous sarcoma virus. I. Intracellular inactivation of cell-transforming ability of Rous sarcoma virus by 5-bromodeoxyuridine and light. J. Virol. **5**:470–477.
2. **Boettiger, D., and H. M. Temin.** 1970. Light inactivation of focus formation by chicken embryo fibroblasts infected with avian sarcoma virus in the presence of 5-bromodeoxyuridine. Nature (London) **228**:622–624.
3. **Colby, C., and G. Edlin.** 1970. Nucleotide pool levels in growing, inhibited, and transformed chick fibroblast cells. Biochemistry **9**:917–920.
4. **Fritsch, E., and H. M. Temin.** 1977. Formation and structure of infectious DNA of spleen necrosis virus. J. Virol. **21**:119–130.
5. **Humphries, E. H., and H. M. Temin.** 1972. Cell cycle-dependent activation of Rous sarcoma virus-infected stationary chicken cells: avian leukosis virus group-specific antigens and ribonucleic acid. J. Virol. **10**:82–87.
6. **Humphries, E. H., and H. M. Temin.** 1974. Requirement for cell division for initiation of transcription of Rous sarcoma virus RNA. J. Virol. **14**:531–546.
7. **Ihle, J. H., F. T. Kenney, and R. W. Tennant.** 1974. Evidence for a stable intermediate in leukemia virus activation in AKR mouse embryo cells. J. Virol. **14**:451–456.
8. **Kang, C.-Y., and H. M. Temin.** 1973. Lack of sequence homology among RNAs of avian leukosis-sarcoma viruses, reticuloendotheliosis viruses, and chicken endogenous RNA-directed DNA polymerase activity. J. Virol. **12**:1314–1324.
9. **Meselson, M., F. W. Stahl, and J. Vinograd.** 1957. Equilibrium sedimentation of macromolecules in density gradients. Proc. Natl. Acad. Sci. U.S.A. **43**:581–588.
10. **Meselson, M., R. Yuan, and J. Heywood.** 1972. Restriction and modification of DNA. Annu. Rev. Biochem. **41**:447–466.
11. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493–497.
12. **Rubin, H., and H. M. Temin.** 1959. A radiological study of cell-virus interaction in the Rous sarcoma. Virology **7**:75–91.
13. **Rush, M. G., and R. C. Warner.** 1970. Alkali denaturation of covalently closed circular duplex deoxyribonucleic acid. J. Biol. Chem. **245**:2704–2708.
14. **Studier, F. W.** 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. **11**:373–390.
15. **Temin, H. M.** 1967. Studies on carcinogenesis by avian sarcoma viruses. V. Requirement for new DNA synthesis and cell division. J. Cell. Physiol. **69**:53–63.
16. **Temin, H. M.** 1968. Carcinogenesis by avian sarcoma viruses. Cancer Res. **28**:1835–1838.
17. **Temin, H. M., and V. K. Kassner.** 1974. Replication of reticuloendotheliosis viruses in cell culture: acute infection. J. Virol. **13**:291–297.
18. **Temin, H. M., and V. K. Kassner.** 1975. Replication of reticuloendotheliosis viruses in cell culture: chronic infection. J. Gen. Virol. **27**:267–274.
19. **Varmus, H. E., T. Padgett, S. Heasley, G. Simon, and J. M. Bishop.** 1977. Cellular functions are required for the synthesis and integration of avian sarcoma virus-specific DNA. Cell **11**:307–319.