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Kinetics of Murine Type C Virus-Specific DNA Synthesis in Newly Infected Cells

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Replicating transforming functions of Rauscher leukemia virus (RLV) and the RLV pseudotype of Moloney sarcoma virus in mouse embryo fibroblasts were found to be most sensitive to inhibition by cytosine arabinoside (ara-C) 30 to 90 min after infection. The initiation of intracellular RLV DNA synthesis was detected by nucleic acid hybridization within this time interval. Treatment of infected cells with cytosine arabinoside abolished RLV DNA synthesis. Peak synthesis of the DNA complementary to the infecting RLV genome, the (−) strand, occurred 40 to 60 min after infection. During this interval two species of DNA were observed with estimated molecular weights of 0.5×10^5 to 1.0×10^5 and 3×10^6. Peak synthesis of the (+) strand viral DNA occurred 50 to 70 min after infection. The initial species detected had a molecular weight of 1.5×10^5 to 4.0×10^5 which shifted as a function of time to 3×10^6. Both (+) strand species were initially detected in the cytoplasm followed by a rapid (10-min interval) appearance of the faster-sedimenting species in the nucleus. The virus-specific (−) and (+) strand DNA species are presumably unintegrated intermediates in provirus formation.

The initial event in the replication in and transformation of cells by RNA tumor viruses is the transcription of the viral RNA genome into DNA (30). This has been supported by considerable indirect evidence involving the effect of inhibitors on DNA synthesis of infected cells (4, 30), detection of RNA-directed DNA polymerase in virions (7, 33), and isolation of biologically active DNA from transformed cells (16). More direct evidence for the synthesis of viral DNA beginning within 1 to 3 h after infection has been obtained by autoradiography (14), isopycnic Cs_2SO_4 centrifugation of incoming viral RNA (19, 28, 29), and nucleic acid hybridization of unintegrated DNA (1, 17, 21, 36).

Recently several reports using single-stranded nucleic acid probes have distinguished two DNA synthetic events. First, a DNA copy synthesized on the RNA template of the infecting genome has been observed (1, 11, 17, 35). If the infecting single-stranded RNA genome is assigned the designation "positive [(+)] RNA strand," then this copy would logically be called the negative [(−)] DNA strand. Second, the complementary strand to the DNA described above must be synthesized after the enzymatic degradation of the RNA template. This DNA would then be called the positive [(+)] DNA strand. Detection of this DNA in unintegrated form has been reported (11, 21, 35).

This report summarizes the results of experiments designed to determine: (i) the critical time for provirus formation using the inhibitor of DNA synthesis cytosine arabinoside; (ii) the kinetics of synthesis of (−) strand DNA by hybridization of excess purified Rauscher leukemia virus (RLV) RNA with tritiated DNA obtained from infected cells; (iii) the kinetics of synthesis of (+) strand DNA monitored by hybridization of the tritiated single-stranded DNA copy of viral RNA with excess unlabeled cellular DNA. The size of both strands was also determined by velocity centrifugation in alkaline glycerol gradients.

MATERIALS AND METHODS

Cell cultures. Mouse embryo fibroblast (MEF) cultures were initiated from 14- to 21-day-old BALB/c embryos obtained from Flow Laboratories, Inc., Rockville, Md. Primary cultures in 32-oz (about 960 ml) bottles were trypsinized and replated on 60- or 100-mm plastic petri dishes in growth medium consisting of Eagle minimal essential medium (Flow Laboratories, Inc.) supplemented with 10% fetal bovine serum.

Treatment of cells for drug inhibition studies. The Rauscher pseudotype of the Moloney murine

sarcoma virus, M-MSV(RLV) (22), was added in 0.5 ml for 15 min at 120 to 150 focus-forming units/60-mm dish to subconfluent cell monolayers (2×10^5 cells). The time of virus application was taken as zero time. At selected intervals of time after infection, 10 μg of 1-β-D-arabinofuranosyl cytosine (ara-C) (Terra-Marine Bioresearch, La Jolla, Calif.) per ml was applied. After either 15 or 30 min at 37 C, the cell monolayer was washed twice with Earle balanced salt solution (EBSS) and then fresh medium was added.

Assays. Incorporation of [*methyl*-^{3}H]thymidine (New England Nuclear Corp.) into trichloroacetic acid-insoluble radioactivity was performed as described by Kennell (18). Transformation was quantitated by scoring the number of foci, using a low-power microscope, 5 to 6 days after infection. The viral polymerase assay was performed on supernatant fluids taken from day 2 to day 4 postinfection. After clarification by low-speed centrifugation, polyethylene glycol 6000 and NaCl were added to supernatant fluids to a final concentration of 6 and 3%, respectively, and shaken for 2 h on a wrist-action shaker. The virus was then pelleted at $7,000 \times g$ for 10 min in a Beckman model J-21 centrifuge. The virus pellet was resuspended to give a 10-fold concentration in 200 μl of 0.01 M Tris buffer, pH 7.4. Reaction mixtures contained in a final volume of 60 μl: 20 μl of virus suspension, 0.04% Nonidet P-40 (NP-40), 0.1 M glycine buffer, pH 8.3, 30 mM NaCl, 1 mM $MnCl_2$, 0.1 mM [^{3}H]TTP (50 Ci/mmol), and oligo(dT) and poly(A) at 10 μg/ml each. Incubations were at 37 C for 30 min. The reaction was terminated with 0.3 ml of 1 N perchloric acid. Seventy-five microliters of calf thymus DNA (1 mg/ml) was added and the mixtures were incubated for an additional 15 min at 4 C. A 0.6-ml amount of cold water was then added, and the tubes were centrifuged at 2,000 rpm for 5 min. The supernatant was aspirated to waste and the precipitate was redissolved in 0.1 ml of 0.2 N NaOH. The procedure beginning with addition of 0.3 ml of 1 N perchloric acid was repeated. Finally, the DNA was precipitated with 3 ml of 10% trichloroacetic acid, the precipitate was collected on membrane filters (Millipore Corp.), and radioactivity was determined in a Beckman LS-250 scintillation counter.

Treatment of cells for hybridization experiments. RLV derived from clarified supernatant culture fluids of an RLV-infected mycoplasma-free JLS V-9 cell line (37) was added in 2 ml of medium for 25 min to cell monolayers (1.2×10^6 cells/dish) at approximately 2×10^6 infectious particles/100-mm dish. The monolayer was washed once with EBSS and then 2 ml of Eagle minimal essential medium supplemented with 10% fetal bovine serum was added. For detection of (+) strand DNA no further additions were made. For detection of (−) strand DNA, 50 μCi of [*methyl*-^{3}H]thymidine per ml (48 to 55 Ci/mmol; New England Nuclear Corp.) was added at selected intervals and allowed to incubate on the monolayers for 20 min at 37 C.

To terminate incubation, the cells were cooled quickly by washing twice with EBSS precooled to 4 C containing 10^{-2} M sodium azide and placing the dishes on cooled trays at 4 C. The cells were scraped off the dish with a rubber policeman, and cells collected from 5 to 10 plates were combined in a cooled plastic centrifuge tube. The cells were packed by centrifugation, washed once with cold EBSS, and resuspended in 200 μl of isotonic saline solution.

In experiments where separation of cytoplasmic from nuclear fractions was performed, a modification of the technique described by Penman (25) was used. To the cells in isotonic saline solution, NP-40 (Shell Chemical Co.) was added to a final concentration of 1% with thorough mixing. After 5 min the crude nuclear preparation was pelleted and the supernatant was removed. The nuclear fraction was resuspended in 200 μl of isotonic saline solution containing a 1% solution of Tween 40 (Sigma Chemical Co.) and sodium deoxycholate (Fisher Scientific Co.). After 5 min the nuclear fraction was again pelleted and the supernatant fraction from this centrifugation was combined with the previous one.

Cell lysis and alkaline glycerol gradient centrifugation. Cell lysis and extraction of undegraded DNA was accomplished by a modification of the methods of Sambrook et al. (26) and Nonoyama and Pagano (23). Either whole-cell suspensions or subcellular fractions (cytoplasmic or nuclear) were gently pipetted into 0.4 ml of lysing solution consisting of 0.5 N NaOH, 1% sodium lauroyl sarcosinate (Chemical Additives Co., Farmingville, N.Y.), and 0.08 M EDTA. The lysing solution had been layered previously on a linear 10 to 30% or 5 to 20% glycerol gradient in 0.1 N NaOH and 0.8 M NaCl, pH 12.2. Gradients were in turn layered over a pure glycerol cushion. Lysis was allowed to proceed for 4 h at 4 C. Single-stranded circular ϕX174 DNA (Miles Laboratories, Inc.) was routinely added to the lysis solution. In parallel gradients, tritium-labeled lambda bacteriophage restriction enzyme fragments of DNA (gift of Richard Roberts, Cold Spring Harbor Laboratory [2, 3]) were used as molecular weight markers.

Alkaline glycerol gradient centrifugation was performed for 10 h at 37,000 rpm in a Spinco SW41 rotor at 4 C. After centrifugation, 0.6-ml fractions were collected starting from the bottom of the tube. Individual fractions in 0.1 N NaOH were heated to 80 C for 5 min to further degrade RNA. The absorbance profile of the gradient at 260 nm was obtained to be certain that overloading had not occurred. DNA in each fraction was precipitated after addition of 20 μg of calf thymus DNA, 0.2 volume of 2.0 M sodium acetate, pH 5.0, and 2.5 volumes of 90% ethanol. After standing overnight at −20 C, the precipitated DNA was centrifuged at 2,000 rpm for 30 min in an IEC model PR-6 centrifuge. The supernatant was removed by aspiration and the pellet was solubilized in 50 μl of 0.1 × SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate). The DNA was stored at −20 C until used for hybridization studies.

Detection of viral (−) strand DNA. An aliquot of the solubilized [^{3}H]DNA was counted so that 600 to 1,000 counts/min could be used in each hybridization. Thus, adjacent gradient fractions were pooled if

individual fractions did not contain enough radioactivity. For each fraction or pool of DNA obtained from infected cells, two hybridization reactions were carried out, one reaction containing added purified RLV 70S RNA and one without added RNA. Hybridization of [^{3}H]DNA from RLV-infected cells was performed for 64 h in 2× SSC with 0.1% sodium dodecyl sulfate at 67 C in a total volume of 100 μl. Analysis for DNA-RNA hybrids was by digestion with single-strand-specific nuclease, S-1, at 45 C for 2 h as lescribed by Okabe et al. (24).

The amount of RLV 70S RNA used (0.2 to 0.4 μg) was calculated to be in vast weight excess of the [^{3}H]DNA. This is based on the specific activity of the [^{3}H]thymidine (~50 Ci/mmol), assuming approximately 10-fold dilution when added to cell culture (15). The amount of newly synthesized DNA represented by 1,000 counts/min thus has an upper limit of ~0.1 ng.

The reaction without 70S RLV RNA was the control to monitor DNA-DNA self-annealing, which ranged from 9 to 16% of the input counts. These control counts were subtracted from the counts obtained from the corresponding hybridization reaction containing 70S RNA to give the values shown in the various figures.

Detection of (+) strand viral DNA. Solubilized DNA was allowed to hybridize with 800 to 1,200 counts/min of single-stranded viral complementary [^{3}H]DNA (cDNA) under conditions similar to that described above. The cDNA was prepared by polymerizing [^{3}H]TTP (40 to 55 Ci/mmol; New England Nuclear Corp.) with unlabeled deoxynucleoside triphosphates in the presence of purified NP-40-treated RLV overnight in the presence of 100 μg of actinomycin D per ml. The specific activity of the in vitro DNA was calculated to be 2×10^7 counts/min per μg. When incubated as described above with 0.2 μg of 70S RLV RNA, 86% of the [^{3}H]cDNA became S-1 nuclease resistant.

RESULTS

Kinetics of inhibition of replication and transformation by ara-C. A requirement of DNA synthesis for replication and transformation activities of RNA tumor viruses has been well established by using DNA inhibitors (4, 5, 30). This DNA synthesis was shown to be virus specific rather than simply stimulation of cellular DNA (6, 8). A critical temporal analysis of viral DNA synthesis was performed by Bader (5), who observed that Rous sarcoma virus progeny formation was inhibited to the highest degree when iododeoxyuridine was applied within 1 h after infection. Our initial experiments were made to define the critical interval in the murine system by using the DNA inhibitor ara-C.

Ara-C has been shown to inhibit DNA synthesis in uninfected animal cell cultures (27) and cell cultures infected by RNA tumor viruses (4, 10, 31). Cells washed free of ara-C have been observed to resume DNA synthesis quickly (9); thus, this agent is particularly suited to determining the timing of proviral DNA synthesis.

MEF were infected with M-MSV(RLV) at low multiplicity (120 to 150 focus-forming units/2 × 10^5 cells). At designated intervals ara-C (10 μg/ml, final concentration) was added. This concentration was found to inhibit total DNA synthesis by 86% when left in contact with the cells for 30 min. After either 15 or 30 min the drug was washed out and the infected cells were reincubated at 37 C. Culture fluids in contact with cells from days 2 to 4 after infection were concentrated 10-fold and assayed for viral polymerase activity. The same dishes were scored for foci on day 5 postinfection. Thus, the capacity to form progeny and transform cells was tested in the same experiment. Using 30-min pulses of ara-C, the most drug-sensitive intervals were found to be 30 to 60 min and 60 to 90 min postinfection for inhibition of both progeny formation and transformation (Table 1, experiment 1). In these intervals both viral functions were inhibited 85 to 90% compared with control values. From 90 min to 3.5 h postinfection, inhibition declined from 70 to 50% for both functions. To further define the critical interval, 15-min pulses of ara-C were used at 30 to 90 min postinfection. It was found (Table 1, experiment 2) that the drug was most inhibitory in the time intervals of 45 to 60 min and 60 to 75 min.

These inhibitor experiments suggested that viral DNA intermediates may be synthesized in the 30- to 90-min postinfection interval. An alternate possibility might be that cellular DNA essential for later viral DNA synthesis is synthesized during this interval. To distinguish between these possibilities, the detection of unintegrated viral DNA sequences was attempted by using nucleic acid hybridization.

Synthesis of viral (−) strand DNA. In the provirus model (32), the initial molecular event is postulated to be the synthesis of the DNA copy of the infecting viral genome, the (−) strand DNA, by the viral RNA-directed DNA polymerase. To detect this intermediate in vivo, MEF infected by RLV at 1 to 2 infectious units per cell were pulse labeled for 20 min with high-specific-activity [^{3}H]thymidine for four intervals after infection (20 to 40, 30 to 50, 40 to 60, and 50 to 70 min). Immediately after the radioactive pulse the cells were washed in cold EBSS, scraped from the dish, and gently lysed on top of alkaline glycerol gradients with detergent in alkaline solution. Cell disruption in this

TABLE 1. *Effect of cytosine arabinoside on cell transformation and progeny formation by M-MSV(RLV)*[a]

Time postinfection (min)	Number of foci/plate	Polymerase (counts/min/ 10-μl aliquot)
Expt 1		
0–30	54	4,305
30–60	16	774
60–90	20	818
90–120	41	2,122
120–150	62	2,783
150–180	72	3,720
180–210	68	4,106
No Drug	138	7,280
Expt 2		
15–30	66	5,109
30–45	42	3,135
45–60	28	1,016
60–75	33	1,180
75–90	47	2,978
90–105	61	4,485
No Drug	143	7,401

[a] 4×10^5 MEF/60-mm petri dish were infected with 125 to 150 focus-forming units of M-MSV(RLV). Ara-C was added to give a final concentration of 10 μg/ml at designated intervals for 15 or 30 min. After removal of ara-C, cells were incubated at 37 C with Eagle minimal essential medium supplemented with 10% fetal bovine serum. Culture fluids of three plates from days 2 to 4 postinfection were clarified by low-speed centrifugation and concentrated by polyethylene glycol precipitation. Polymerase activity was assayed as described in the text. Assays of supernatant fluids from uninfected cells were taken as blanks (average 340 counts/min) and subtracted from the values given above. Foci were scored on day 5 postinfection.

way efficiently prevents cellular nuclease activity and eliminates shearing of DNA. After centrifugation the gradients were fractionated. Tubes 1 through 9 (higher molecular weight) were pooled, and tubes 10 through 18 (lower molecular weight) were pooled, neutralized, and precipitated with ethanol.

The higher-molecular-weight pools for all time intervals showed no detectable hybridization with purified RLV RNA. A histogram of the low-molecular-weight pools (Fig. 1) shows that RLV (−) strand DNA sequences were detectable and that peak synthesis of (−) strand DNA occurred in the 40- to 60-min interval. Uninfected MEF cells pulsed for 20 min showed no detectable (−) strand DNA sequences. In addition, infected cells treated with ara-C (10 μg/ml) and pulsed with [^{3}H]thymidine between 40 and 60 min postinfection showed less than 10% of the untreated hybridization value (data not shown).

The position of viral DNA sequences in alkaline glycerol gradients was then determined. RLV-infected MEF cultures were pulse labeled with [^{3}H]thymidine 40 to 60 min after infection. The cells were scraped from 100-mm plates immediately and lysed as described above. To hybridize the DNA of each fraction without pooling, 8×10^6 to 10×10^6 infected cells were lysed on each gradient and centrifuged. In parallel gradients, selected endonuclease R·*Hpa*II and endonuclease R·*Eco*R1 lambda bacteriophage digestion products (gift of Richard Roberts, Cold Spring Harbor Laboratories) were centrifuged. The molecular weight of these fragments has been determined by electron microscopy (2, 3).

Over 90% of the [^{3}H]thymidine was incorporated into fractions 1 through 9 (high-molecular-weight DNA). On the other hand, all of the

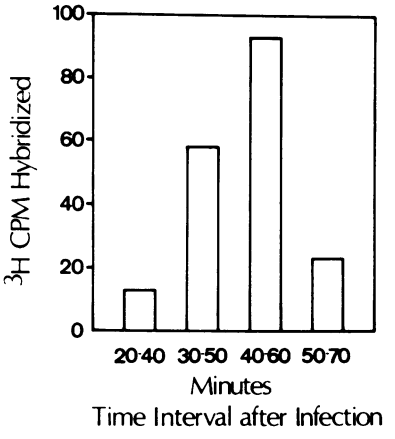

FIG. 1. *Hybridization of pooled MEF cellular [^{3}H]DNA synthesized at four intervals after infection by RLV. Detection of (−) strand viral DNA. Approximately 6.0 × 10^6 cells were gently lysed as described in the text and the DNA was centrifuged in a 10 to 30% linear alkaline glycerol gradient for 10 h at 37,000 rpm in a Spinco SW41 rotor at 10 C. The gradient was fractionated from the bottom; fractions 1–9 and 10–18 were pooled and DNA was precipitated as described in the text. After solubilization, fixed amounts of ^{3}H-labeled cellular DNA (600 counts/min) were incubated in 2× SSC with excess RLV 70S RNA for 64 h at 67 C. The mixtures were analyzed for hybridized [^{3}H]DNA with S-1 nuclease. An equal amount of ^{3}H-labeled cellular DNA was incubated without viral RNA and analyzed with S-1 nuclease. This control value monitored DNA-DNA self-annealing, which ranged from 9 to 16% of the input counts. The hybridization values of the fraction 10–18 pool in the figure are net counts obtained after subtraction of the control values.*

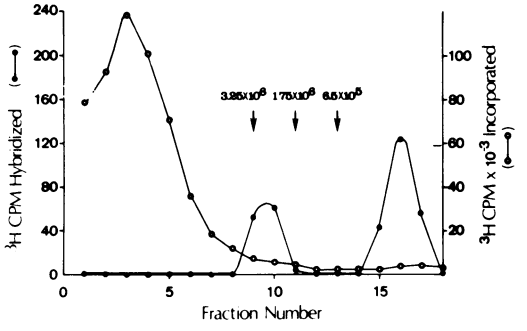

FIG. 2. *Ten to 30% alkaline glycerol gradient sedimentation of DNA synthesized in MEF 40 to 60 min after infection by RLV. Molecular weight analysis of (−) strand viral DNA. 8 × 10⁶ to 10 × 10⁶ cells were lysed and the DNA was centrifuged in a linear 10 to 30% alkaline glycerol gradient for 10 h at 37,000 rpm in a Spinco SW41 rotor at 10 C. The direction of sedimentation is from right to left. In parallel gradients the following marker DNAs were centrifuged: lambda bacteriophage DNA fragment (2+2') of endonuclease R·HpaII digestion, 6.5 × 10⁵ daltons (2); and lambda bacteriophage DNA fragments 1' and 4 of endonuclease R·EcoR1 digestion, 3.3 × 10⁶ and 1.8 × 10⁶ daltons, respectively (3). The gradient was fractionated from the bottom and each fraction was precipitated as described in the text. After solubilization of the precipitate, an aliquot was precipitated with trichloroacetic acid to measure the counts per minute incorporated (○). Hybridization was performed on another aliquot as described in Fig. 1 and measured in tritiated counts per minute hybridized (●).*

virus-specific DNA was found between fractions 9 and 18. Approximately one-third of the virus-specific DNA was found in fractions 9 and 10. Based on the position of the bacteriophage lambda fragment markers, the molecular weight of this fraction was estimated to be 2.3 × 10^6 to 3.3 × 10^6. The remaining two-thirds of the virus-specific DNA sedimented near the top of the gradient with an estimated molecular weight lower than the 6.5 × 10^5 marker. In a later experiment the cells were lysed and sedimented in a 5 to 20% alkaline glycerol gradient in order to determine more exactly the molecular weight of the smaller virus-specific DNA (Fig. 3). This virus-specific DNA sedimented to approximately the same position as complementary viral DNA synthesized in vitro (see Materials and Methods) and slightly slower than the 10^5 lambda bacteriophage marker.

Synthesis of viral (+) strand DNA. Viral (+) strand DNA has been detected in newly infected MEF (21) and duck cells (35). To determine the kinetics of synthesis of this strand, MEF were infected with RLV at a multiplicity of 1 to 2 infectious units per cell. At four time intervals after infection (40, 50, 60, and 70 min), incubation was terminated and the cells were scraped, lysed, and centrifuged in alkaline glycerol gradients as in the previous experiment. Since viral genomic RNA would interfere in the detection of viral (+) strand DNA, careful attention was devoted to washing the cells and treating the DNA in alkaline solutions at elevated temperature to degrade residual RNA (see Materials and Methods). Each gradient fraction was hybridized with complementary [^{3}H]DNA synthesized in vitro from purified detergent-treated RLV.

At 40 min postinfection S-1 nuclease-resistant [^{3}H]DNA was found only at the bottom of the gradient. This pattern probably represents endogenous virus integrated into the host cell genome and is similar to that observed with uninfected MEF (21) (Fig. 4A). At 50 min postinfection (Fig. 4B) RLV-specific DNA was found in fractions 9 to 11 and 12 to 15, the latter containing the predominant amount. By 60 min postinfection (Fig. 4C) RLV-specific DNA was was found to sediment in both fractions 9 to 11 and 12 to 15. At 70 min postinfection (Fig. 4D) most of the virus-specific DNA sedimented in fractions 9 to 11. These data would suggest: (i) that viral (+) strand DNA is synthesized within

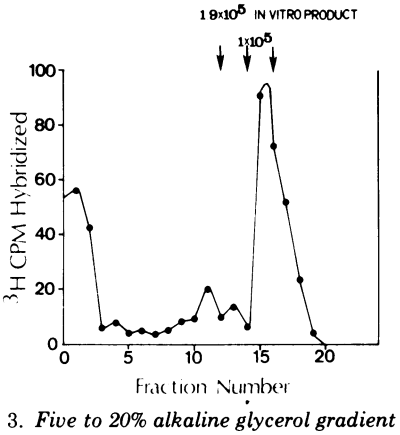

FIG. 3. *Five to 20% alkaline glycerol gradient sedimentation of DNA synthesized in MEF 40 to 60 min after infection by RLV. Molecular weight analysis of (−) strand viral DNA. 7 × 10⁶ cells were lysed and the DNA was centrifuged in a linear 5 to 20% alkaline glycerol gradient for 10 h at 37,000 rpm in a Spinco SW41 rotor at 10 C. In parallel gradients the following marker DNAs were centrifuged: lambda bacteriophage DNA fragments 10 and 21 of endonuclease R·HpaII digestion, 1.9 × 10⁵ and 1 × 10⁵ daltons, respectively (2). After ethanol precipitation of each fraction, the pellet was solubilized and hybridization was performed as described in Fig. 1.*

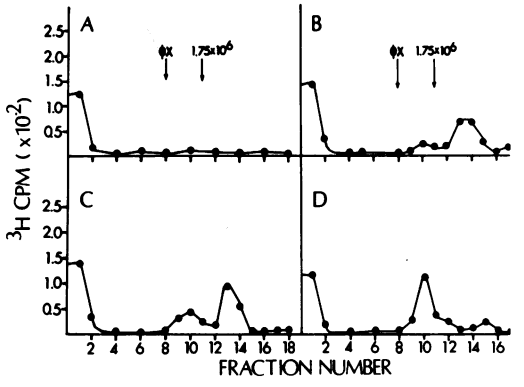

FIG. 4. *Hybridization of cellular DNA fractions with ³H-labeled single-strand DNA endogenous product of RLV polymerase. Time sequence of (+) strand viral DNA synthesis. Cell lysis (4 × 10⁶ cells) and centrifugation were similar to that in Fig. 1. The gradient was fractionated from the bottom and each fraction was precipitated as described in the text. Fixed amounts of ³H-labeled single-strand DNA (750 counts/min) were incubated with cellular DNA in excess (as determined by absorbancy at 260 nm) for 64 h. The hybridized [³H]DNA was assayed with S-1 nuclease. ³H-labeled single-strand DNA incubated without cellular DNA gave an average background value of 96 counts/min. All values shown in the figure are after subtraction of the background value. (A) 40 min postinfection; (B) 50 min postinfection; (C) 60 min postinfection; and (D) 70 min postinfection.*

the critical ara-C inhibitory interval of 30 to 90 min postinfection; and (ii) that the slower-sedimenting viral (+) strand is a precursor to the faster-sedimenting form.

Varmus et al. (35) have reported that (+) strand DNA can be synthesized in infected enucleated duck cells. On the other hand, Sveda et al. (28) and Leis et al. (19) found only unintegrated RNA-DNA viral hybrid molecules, suggesting that (+) strand DNA synthesis is not essential for proviral DNA formation. Using the RLV-infected MEF system described above, we scraped the cells at 52 and 62 min postinfection. At 52 min the major portion of the (+) strand viral DNA was found in the cytoplasm in two peaks (Fig. 5). At 62 min most of the DNA was found in the nucleus only in the faster-sedimenting form.

In a previous report (21) the molecular weight of the faster-sedimenting viral (+) strand DNA was determined to be 2.3×10^6 to 3.3×10^6. The molecular weight of the slower-sedimenting form was determined here by centrifugation in a 5 to 20% linear alkaline glycerol gradient with lambda bacteriophage fragment markers and cDNA synthesized in vitro from purified detergent-treated RLV. This viral (+) strand DNA sedimented faster than the in vitro product and is estimated to be 1.5×10^5 to 4.0×10^5 daltons (Fig. 6).

DISCUSSION

Both (+) and (−) strand RLV-specific DNA species were detected 40 to 70 min after infection in RLV-infected MEF. The inhibitor of DNA synthesis, ara-C, was found to be most inhibitory for RLV progeny formation during a similar interval: 30 to 90 min postinfection. This timing is in substantial agreement with the results of Takano and Hatanaka (29), demonstrating that the [³H]RNA of RLV showed a shift under nondenaturing conditions toward the RNA-DNA hybrid region in Cs_2SO_4 gradients between 45 and 70 min after infection. Since (−) strand viral DNA could not be detected in the presence of ara-C, the drug most likely inhibited the DNA synthetic step catalyzed by the viral RNA-directed DNA polymerase. This conclusion is supported by the in vitro inhibition of the enzyme in the presence of ara-C 5′-triphosphate (34) and the in vivo inhibition of DNA formation on a viral RNA template in the presence of ara-C (29).

Maximal inhibition of RNA tumor virus infection at similar time intervals has been observed with other drugs. Bader (5) found that iododeoxyuridine greatly reduced virus progeny formation in RSV-infected chick cells when

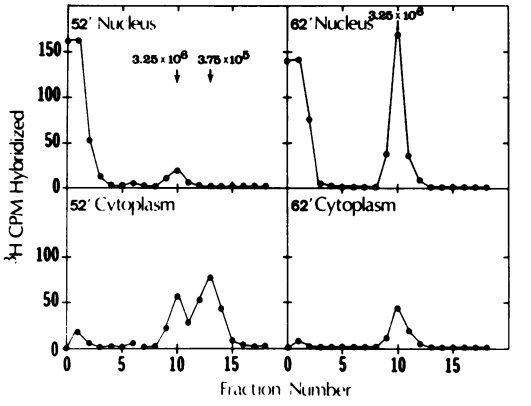

FIG. 5. *Hybridization of cellular DNA fractions with ³H-labeled single-strand DNA product of RLV polymerase. Subcellular site of (+) strand viral DNA. 7 × 10⁶ cells were scraped and separated into nuclear and cytoplasmic fractions by using the double-detergent technique of Penman (25) (see text). These fractions were lysed on separate gradients and subsequent centrifugation was as described in Fig. 1. Gradient fractionation and hybridization were performed as in Fig. 4. The separated fractions of cells harvested 52 and 62 min after infection are shown on the left- and right-hand portions, respectively.*

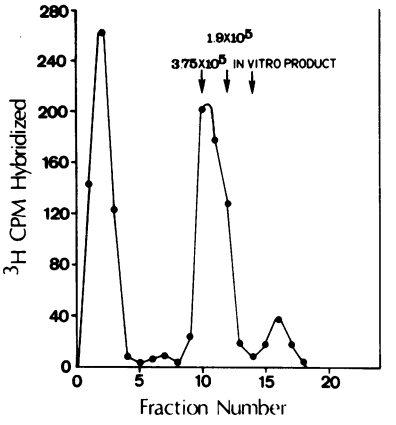

FIG. 6. *Five to 20% alkaline glycerol gradient sedimentation of DNA extracted from RLV-infected MEF 55 min after infection. Molecular weight analysis of (+) strand viral DNA. At 55 min postinfection, 4 × 10⁶ cells were scraped, lysed, and centrifuged for 10 h at 37,000 rpm in a Spinco SW41 rotor at 10 C. In parallel gradients the following marker DNAs were centrifuged: lambda DNA fragments 5 and 10 of endonuclease R·HpaII digestion, 3.8 × 10⁵ and 1.9 × 10⁵ daltons, respectively (2); and single-strand DNA in vitro product of the endogenous RLV polymerase reaction (see text for preparation). After ethanol precipitation of each fraction, the pellet was solubilized and hybridization was performed as described in Fig. 4.*

introduced within 1 h after infection. Cordycepin (20) and hydroxyurea (G. C. Lovinger, in preparation) were found to inhibit cell transformation by M-MSV(RLV) when added 30 to 90 min after infection. The replication of M-MSV(RLV) was inhibited by actinomycin D when added within 2.5 h of infection (12).

In sum, this report and others listed above indicate the critical nature of viral DNA synthesized 1 to 2 h after infection. Yet several laboratories, including our own (Lovinger, in preparation), have found that viral DNA synthesis continues after this time interval. Hatanaka et al. (14) and Kakefuda et al. (17) found that viral DNA synthesis in H-MSV-infected mouse cells continued up to 10 h postinfection. Ali and Baluda (1) found that synthesis of virus-specific DNA continues up to 36 h in chick embryo fibroblasts infected with Rous sarcoma virus. The significance of this later synthesis is unclear at present.

As early as 52 min after infection (+) strand viral DNA was found in the cytoplasm. Ten minutes later most of it was found in the nucleus. Thus the (+) strand viral DNA is synthesized in the ctyoplasm and transported rapidly to the nucleus. Varmus et al. (35), using enucleated duck cells, also found (+) strand viral DNA in the cytoplasm shortly after infection with the B77 strain of avian sarcoma virus.

Virus-specific DNA of molecular weight lower than 3×10^6 (viral RNA subunit weight) has been observed transiently in both (+) and (−) strand viral DNA preparations. Kadefuda et al. (17), Takano and Hatanaka (29), and Guntaka et al. (13) have also observed virus-specific DNA smaller than 3×10^6 daltons. This would suggest that synthesis proceeds in a discontinuous manner. Since in vitro synthesis catalyzed by the viral RNA-directed DNA polymerase produces only small viral DNA, cellular enzymes or host modification of viral enzymes may be involved in viral DNA synthesis. Pulse-chase experiments (unpublished data) show that the 3×10^6-dalton viral DNA as a function of time becomes a faster-sedimenting form in alkaline and neutral glycerol gradients. Guntaka et al. (13) and Gianni et al. (11) have identified a faster-sedimenting viral DNA as a closed circular form. This further suggests that these unintegrated virus-specific DNA species are intermediates in the formation of chromosomal proviral DNA.

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