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RNA-Dependent DNA Polymerase Activity of RNA Tumor Viruses

V. Rous Sarcoma Virus Single-Stranded RNA-DNA Covalent Hybrids in Infected Chicken Embryo Fibroblast Cells

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RNA-DNA covalent hybrids containing viral RNA have been isolated from nuclear fractions of Rous sarcoma virus-infected chicken embryo fibroblast cells shortly after virus infection. The formation of covalent hybrid structures depends upon a functional reverse transcriptase in vivo, since its appearance in cells is temperature dependent when infected with Rous sarcoma virus mutant LA335, which contains a temperature-sensitive reverse transcriptase.

RNA tumor viruses appear to replicate via a DNA intermediate. This conclusion is supported by observations that virus infection is blocked by inhibitors of DNA synthesis (1, 14, 17), the presence of an enzyme capable of transcribing viral RNA into DNA, RNA-dependent DNA polymerase in virions (2, 15), the isolation of a "DNA provirus" in cells (H. Varmus et al., Cold Spring Harbor Symp. Quant. Biol., in press), and the demonstration that DNA isolated from transformed cells is biologically active (6) (H. M. Temin and G. Cooper, Cold Spring Harbor Symp. Quant Biol., in press).

Purified RNA-dependent DNA polymerase catalyzes repair-like DNA synthesis with RNA, DNA, and RNA-DNA hybrids. The enzyme requires primer strands containing 3′ OH termini, which serve as initiation sites for DNA synthesis, and a template strand, which directs DNA synthesis (5, 7, 8, 11, 13, 16). The product of the reaction with AMV RNA is an RNA-DNA covalent hybrid (8, 16) in which newly synthesized DNA is small (5 to 7*S*, as shown by alkaline sucrose gradient analysis).

In vitro studies with reverse transcriptase suggest that viral DNA may be synthesized in cells via a RNA-DNA covalent hybrid. The presence of such a molecule was initially described in Rous sarcoma virus (RSV)-infected chicken embryo fibroblast (CEF) cells (J. Leis et al., 34th Annu. Biol. Colloq., Oregon State Univ., in press) and has also been reported in Rauscher leukemia virus- (T. Tankano, and M. Hatanaka, in press) and Friend leukemia virus-infected (12) mouse cells. In this communication, we describe the characteristics of isolated RNA-DNA covalent hybrid molecules from RSV-infected CEF cells and demonstrate that their appearance in nuclear cell fractions requires a functional reverse transcriptase. This has been demonstrated by using a mutant of RSV, LA335 (9), in which the reverse transcriptase was shown to be temperature sensitive (9; D. Baltimore et al., Proc. Nat. Conf. Virol. Immun. Human Cancer, in press). The hybrid molecules are present in the nucleus of cells infected at permissive temperature but in markedly lower levels in cells infected at non-permissive temperatures.

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MATERIALS AND METHODS

RSV mutant LA335 was a generous gift of P. Vogt and W. Mason, University of Southern California. All other materials were obtained as described previously (8).

Labeling of RSV. RSV (Prague, subgroup C) was grown on CEF monolayers (C/O cells). Virus was labeled by incubating infected cells for 12 to 15 h with 10 mCi of [^{3}H]uridine (2×10^4 counts/min per pmol) per 60 ml of growth media as described by Cheung et al. (3). After this period, the media was removed and cells were washed twice with unlabeled growth media; washed cells were then incubated with 50 ml of growth media, and virus was harvested at 2-h intervals. Cells were removed by centrifugation at $800 \times g$ for 10 min, and the supernatant, containing virus (5×10^6 focus-forming units [FFU]/ml, containing 20 counts/min/pmol of RNA nucleotide), was used to infect cells. LA335 RSV was grown on CEF monolayers (C/O cells) as described by Linial and Mason (9). Virus was labeled and harvested as described above, except that [^{3}H]-labeled virus (stored for 3 days at 4 C on ice) was divided into two portions, one of which was incubated

1 h at 37 C and the other at 41 C in growth media prior to cell infection. This procedure resulted in the inactivation of 43% of the reverse transcriptase at 41 C (77% after 2 h), with only 11% inactivation at 37 C (22% after 2 h) measured by deoxyadenylate-deoxythymidylate co-polymer synthesis with virions activated with Triton X-100 (8).

Preparation of cell fractions. CEF primary cultures (C/O) grown to confluency (4 days post seeding), were trypsinized and seeded at 20×10^6 cells/25 ml in petri dishes (150 by 25 mm). After 1 to 3 h incubation at 37 C in a 5% CO_2 humidified atmosphere, media was removed and each plate was treated with 5 ml of labeled virus suspension containing unlabeled uridine (2×10^{-6} M). After 30 min, fresh growth media was added and cells were incubated for times indicated. Monolayers were washed and trypsinized (0.25% trypsin for 10 s), and cells were suspended in growth media and pelleted at $400 \times g$ for 5 min. Cells were washed twice and then resuspended in a solution containing 0.01 M Tris-hydrochloride, pH 7.4, 0.01 M NaCl, and 1.5 mM $MgCl_2$ (RSB) at a concentration of 2×10^7 to 4×10^7 cells/ml. After 15 min on ice, cells were gently homogenized five to ten times in a tight-fitting homogenizer in 1% Tween 40 and 0.5% deoxycholate. Nuclei were pelleted at $800 \times g$ for 2.5 min, suspended in 2 ml of RSB containing the detergent, pelleted, and frozen at −20 C. The supernatant obtained after pelleting of nuclei was used to isolate cytoplasmic nucleic acid fractions. Such supernatants were adjusted to 0.01 M EDTA and 1% sodium dodecyl sulfate and treated with 2 volumes of 95% ethanol. After centrifugation, pellets were dissolved in a solution containing 0.5% sodium dodecyl sulfate, 0.01 M Tris-hydrochloride, pH 7.4, 0.01 M NaCl, and 1 mM EDTA and frozen.

Isolation of RNA-DNA covalent hybrids by equilibrium Cs_2SO_4 banding centrifugation. Nucleic acid was extracted from nuclei and cytoplasmic fractions by a modification of the Penman procedure (10). Nuclei fractions were suspended in 0.8 ml of a solution containing 1% sodium dodecyl sulfate, 50 mM EDTA and treated with 1 volume of phenol (neutralized and saturated with water) at 60 C for 2 min. One volume of 1% isoamyl alcohol in chloroform was added, and the mixture was incubated at 60 C for 2 min. After centrifugation, the phenol-chloroform phase was removed, and the aqueous phase was extracted three times each with an equal volume of 1% isoamyl alcohol in chloroform at 25 C. The aqueous phase was collected, adjusted to 10 mM EDTA, 25 mM sodium phosphate (pH 7), heated at 100 C for 3 min and then placed in ice. The cooled solution was treated with 0.4 ml of 10× SSC (SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) and 0.01 ml of 10% Sarkosyl, and the mixture was diluted to 4 ml. Solid Cs_2SO_4 (2.8 g) was added, yielding a refractive index of 1.3780, and the mixture was centrifuged for 67 h at 32,000 rpm in a Spinco SW65 rotor at 22 C. Eighteen-drop fractions were collected from a hole pierced in the bottom of the tube, and refractive indexes were determined.

RESULTS

Various times after infection nuclei and cytoplasmic fractions were prepared from CEF infected with [^{3}H]uridine-labeled RSV. The nucleic acid in each fraction was isolated by a modification of the phenol-sodium dodecyl sulfate method described by Penman (10) and analyzed by isopycnic banding in Cs_2SO_4. In cell fractions prepared from 2×10^6 and 2.6×10^6 cells (averaging the results of two experiments) 6 h after infection with [^{3}H]RSV, 89% of the radioactivity (4.7×10^6 counts/min) was recovered in the cytoplasmic fraction, whereas 11% of the label (5.2×10^5 counts/min) was recovered in the nuclear fraction. Of label recovered in the cytoplasmic fraction, 90% banded at the density of free RNA in Cs_2SO_4 after heat treatment at 100 C for 3 min. The remaining radioactivity was distributed throughout the hybrid and DNA density regions without forming a distinct peak. When the latter material was isolated, dialyzed, heated 2 min at 100 C or 15 min at 65 C in the presence of 3% formaldehyde, and then rebanded in Cs_2SO_4, the label now banded as free RNA, suggesting that this labeled material was not covalently linked to DNA. Thus, stable covalent RNA-DNA structures were not detected in the cytoplasmic fraction of CEF cells.

A different distribution of radioactivity was observed with material that was isolated from nuclei 6 h after infection, treated with 3% formaldehyde for 15 min at 65 C, and subjected to isopycnic Cs_2SO_4 gradient centrifugation (Table 1). Of the total tritium recovered, 33% banded in the RNA region (between densities 1.6 to 1.7 g/cm^3), 21% in the hybrid region (density between 1.5 to 1.6 cm^3), and the

TABLE 1. *Distribution of [^{3}H]nucleic acid from nuclei after isopycnic formaldehyde Cs_2SO_4 gradient centrifugation*[a]

Time after infection (h)	Distribution of ^{3}H label (%)		
	RNA	Hybrid	DNA
3	38	33	29
6	33	21	46
9	34	9	57

[a] CEF (1.2×10^6 cells) were infected with tritium-labeled RSV at a multiplicity of infection of 1. At various times after infection, cell nuclei were prepared from 4×10^6 cells, and the nucleic acid was isolated. The recovery of the tritium label after infection for 3, 6, and 9 h was 20,900, 48,073, and 78,430 counts/min, respectively. Each fraction was treated with 3% formaldehyde for 15 min at 65 C and subjected to isopycnic Cs_2SO_4 gradient centrifugation. The amount of radioactivity banding between densities of 1.5 to 1.6 g/cm^3 was taken as RNA-DNA hybrid. Recovery of radioactivity from the Cs_2SO_4 gradients was 60%.

remainder in the DNA region (density between 1.4 to 1.5 g/cm³). The material recovered from the hybrid region rebanded at the same density and thus represented labeled RNA covalently linked to unlabeled DNA. The label initially isolated in the RNA region (density 1.6 to 1.7 g/cm³) was contained in RNA, whereas the label banding in the DNA region (1.40 to 1.48 g/cm³) was mostly DNA. The latter material was 91% sensitive to DNase digestion and 89% resistant to RNase. Exhaustive digestion of this material with snake venom phosphodiesterase and pancreatic DNase liberated [³H]dCMP as analyzed by paper chromatography (NH_4OH-isopropanol-borate) and paper electrophoresis at pH 3.5. Thus, it appears that part of the labeled viral RNA was degraded to mononucleotides, reduced to deoxyribonucleotides, and utilized for DNA synthesis. Approximately 10% of the material isolated in the DNA region is sensitive to RNase and alkali digestion, indicating the presence of some short [³H]RNA sequences covalently linked to large DNA.

The relative amount of radioactivity recovered (after denaturation with formaldehyde) as RNA-DNA covalent hybrid decreased, whereas the label recovered in the DNA region increased with time after infection (Table 1). In addition, the amount of hybrid recovered in nuclei 5 h postinfection was proportional to a multiplicity of infection between 0.02 and 2.

Properties of isolated RNA-DNA hybrid. To characterize the RNA-DNA covalent hybrid, it was purified by isopycnic Cs_2SO_4 gradient centrifugation after heat denaturation without formaldehyde treatment. The material recovered in the hybrid region (1.5 to 1.6 g/cm³) was again heat denatured and rebanded in isopycnic Cs_2SO_4 gradients (see Fig. 1). The label, isolated in the hybrid region (density, 1.57 g/cm³) was still in RNA since it was completely sensitive to RNase digestion and insensitive to DNase (7%). The RNA possessed single-stranded structure, since this material was completely susceptible to attack by nuclease SI. If the RNA-DNA covalent hybrid material was heated at 100 C for 2 min and banded a third time, it still banded at a density of 1.57 g/cm³. Treatment of hybrid material with DNase shifted the banding density of label to 1.61 g/cm³. The DNase-treated material was hybridized to an excess of [³²P]dTMP-labeled DNA prepared from RSV by reverse transcriptase; 55% of the labeled RNA hybridized to the transcript DNA as measured by its resistance to nuclease SI, an enzyme specific for single-stranded structure. Furthermore, material resistant to nuclease SI banded in the hybrid region when analyzed by Cs_2SO_4 banding centrifugation, suggesting that a substantial portion of the RNA in the RNA-DNA covalent hybrid represented viral RNA sequences.

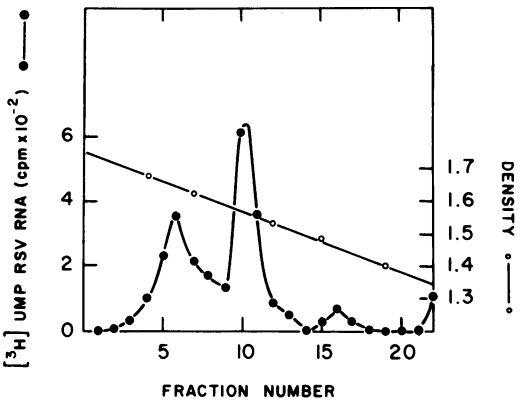

FIG. 1. *Equilibrium Cs_2SO_4 density gradient centrifugation of [³H]UMP-labeled RSV RNA isolated from the nucleus fraction of cells. A virus suspension (5 ml) of RSV (Prague, subgroup C), labeled with [³H]uridine (5 × 10⁶ FFU/ml), was used to infect 2 × 10⁷ cells. After 6 h of incubation, cell fractions were prepared. Nuclei fractions contained 1.65 × 10⁵ acid-insoluble counts/min, whereas cytoplasmic fractions contained 3.71 × 10⁶ acid-insoluble counts/min. Nucleic acid was extracted from the nuclei fractions (1.59 × 10⁵ counts/min in 1.3 ml) and subjected to equilibrium Cs_2SO_4 gradient centrifugation. Tritium label (in counts per minute) was distributed as follows: RNA region (density 1.69 to 1.63 g/cm³), 3.95 × 10⁴; hybrid region (density 1.63 to 1.49 g/cm³), 6.63 × 10⁴; DNA region (density 1.49 to 1.43 g/cm³), 2.86 × 10⁴. The material banding between the density of 1.63 to 1.49 g/cm³ was pooled and dialyzed overnight at 4 C against 1 l of 10 mM sodium phosphate (pH 7) and 1 mM EDTA, brought to a final volume of 4 ml containing 35 mM sodium phosphate (pH 7), 11 mM EDTA, 0.025% Sarkosyl, heated at 100 C for 3 min, and cooled in ice. Cs_2SO_4 was added as above, and the mixture was centrifuged at 30,000 rpm in a Spinco SW50.1 rotor at 20 C for 64 h. Fractions were collected as above, refractive indexes were measured, and 0.01-ml aliquots were precipitated with trichloroacetic acid and counted. Recovery of label from the Cs_2SO_4 gradient was 85%. Symbols: Tritium-labeled RSV RNA, ○; density, ●.*

The approximate molecular weight of the RNA-DNA hybrid molecules isolated after extraction from nuclei avoiding extensive shearing was determined by sucrose gradient velocity centrifugation in 80% dimethyl sulfoxide using φX174 viral DNA (1.7 × 10⁶) as a molecular weight marker (Fig. 2). In these experiments, [³H]-labeled nucleic acid was first sized by di-

methyl sulfoxide gradient centrifugation. The amount of RNA-DNA covalent hybrid molecules present in each size class was then determined by isopycnic gradient centrifugation (Table 2). Of the total amount of radioactivity in hybrid molecules, less than 3% sedimented twice as fast as ϕX174 viral DNA, 21% sedimented one and one-half times as fast as ϕX DNA, 37% with ϕX DNA, and 39% sedimented slower than ϕX DNA. Thus, 58% of the hybrid molecules contained a molecular weight varying between 10^6 to 2.5×10^6.

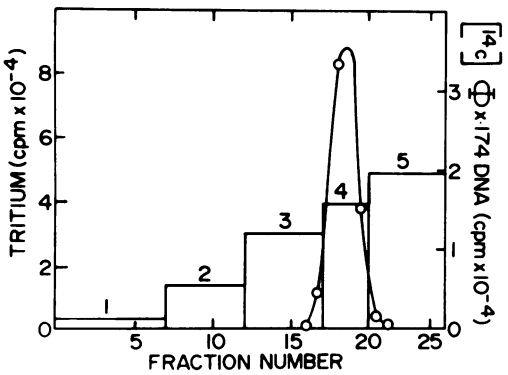

FIG. 2. *Sucrose gradient centrifugation (80% dimethyl sulfoxide) of nuclear nucleic acid. Nuclei were prepared from 5.4 x 10^6 cells 4.5 h after infection with tritium-labeled RSV (multiplicity of infection, 1). Nucleic acid was extracted as described in Materials and Methods, except that after extraction with chloroform-isoamyl alcohol at 60 C 0.2 ml of 10% sodium dodecyl sulfate was added, and the aqueous phase (3 ml) was dialyzed against 500 ml of 0.2% sodium dodecyl sulfate, 0.1 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA overnight at 25 C. The dialyzed fraction was then extracted three times with an equal volume of chloroform-1% isoamyl alcohol, and the nucleic acid was precipitated with the addition of 2 volumes of ethanol at −20 C. The precipitate was collected and dissolved in 1 ml of 0.05 M NaCl, 10 mM sodium phosphate (pH 7.0), 10 mM LiCl by rotating gently on a multipurpose rotator at 4 C overnight. An insoluble precipitate was removed by centrifugation at 600 x g for 2 min in a clinical centrifuge, and the supernatant containing 139,000 counts/min was layered onto a 9.8-ml linear 0 to 20% sucrose gradient in 80% dimethyl sulfoxide, 10 mM LiCl, 10 mM Tris (pH 7.0), 1 mM EDTA. After centrifugation at 36,000 rpm in a Spinco SW41 rotor at 25 C for 19 h, fractions of 35 drops were collected from a hole pierced in the bottom of the tube, and the amount of radioactivity was determined. [^{14}C] ϕX174 viral DNA was run as a standard in a separate gradient. The recovery of radioactivity (counts per minute) in fractions 1 to 5 was as follows: 2,670, 13,240, 29,700, 39,000, and 49,000, respectively. [^{14}C] ϕX174 DNA, O; bar graph indicates total counts per minute per fraction. The direction of sedimentation was from right to left.*

TABLE 2. *Distribution of [^{3}H]nucleic acid isolated from dimethyl sulfoxide sucrose gradients after equilibrium Cs_2SO_4 gradient centrifugation*[a]

Dimethyl sulfoxide gradient fraction analyzed	Percent of tritium banding at density of		
	RNA	Hybrid	DNA
2	15	10	75
3	46	30	24
4	34	45	21
5	54	31	15

[a] Tritium-labeled nucleic acid isolated from dimethyl sulfoxide sucrose gradient fractions 2 to 5 (see Fig. 2) were precipitated by the addition of 2 volumes of ethanol. After sitting at −20 C overnight, the precipitates were collected, dissolved in 4 ml of 2.5 mM EDTA, 12 mM sodium phosphate (pH 7.0), 0.025% Sarkosyl, heated at 100 C for 2 min, and subjected to isopycnic Cs_2SO_4 gradient centrifugation. The recovery of radioactivity after gradient centrifugation for fractions 2 to 5 was 10,200, 25,500, 29,700, and 46,000, respectively. The amount of acid-insoluble radioactivity banding at densities of RNA region (1.6 to 1.7 g/cm^3), hybrid region (1.5 to 1.6 g/cm^3), and DNA region (1.4 to 1.5 g/cm^3) is expressed as percent of total radioactivity recovered.

Presence of viral [^{3}H]RNA-DNA covalent hybrid in RSV LA335-infected cells. A temperature-sensitive mutant of RSV, LA335, isolated by Linial and Mason (9), contains a temperature-sensitive lesion in reverse transcriptase (9; Baltimore et al., Proc. Nat. Conf. Virol. Immun. Human Cancer, in press). This mutant provides a functional test to determine whether formation of hybrid molecules depends upon the action of reverse transcriptase. Labled viral RNA-DNA covalent hybrid molecules can be detected, after banding in a formaldehyde-Cs_2SO_4 density gradient, in nuclear fractions 6 h postinfection with [^{3}H]RSV LA335 at 37 C but in greatly reduced amounts at 2 (not shown), 4, and 6 h postinfection at 41 C (Fig. 3). The labeled material isolated at 37 C which banded at the density of 1.44 was 12% sensitive to pancreatic RNase, whereas that formed at 41 C was less than 0.2% sensitive. This material represents small pieces of RNA covalently linked to large DNA. All label banding at heavier densities at both temperatures was 100% sensitive to RNase. In a separate experiment, a spontaneous revertant of LA335, isolated from stocks of LA335 which had lost its temperature sensitivity, was used to infect CEF cells at 37 and 41 C (Fig. 4). At both temperatures, covalent hybrid molecules were observed. Hybrid molecules were also detected in nuclei of CEF cells 4.5 h postinfection with wild-type RSV at 41 C. These experiments indicate

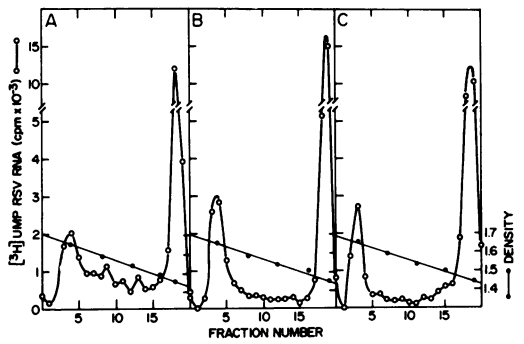

FIG. 3. *Equilibrium Cs_2SO_4-formaldehyde density gradient centrifugation of [^3H]uridine-labeled LA335 RNA isolated from nuclei of infected cells. CEF cells (1.95 × 10^7/dish) were incubated 60 min at 37 C after trypsinization and then allowed to stand 10 min at 37 and 41 C before infection with [^3H]LA335 RSV. Virus (25 ml) was allowed to adsorb 40 min at each respective temperature before being replaced by growth media. Virus titers were 4 × 10^5 FFU/ml at 37 C and 4 × 10^3 FFU/ml at 41 C. Four and six hours after infection, cell fractions were prepared, and nucleic acid was isolated, treated with 3% formaldehyde for 15 min at 65 C, and subjected to equilibrium Cs_2SO_4 gradient centrifugation as described in Materials and Methods, except that 3% formaldehyde was included in the gradients. Radioactivity recovered (in counts per minute) in nuclear fractions was as follows: at 37 C, 5.6 × 10^4 (6 h); at 41 C, 3.86 × 10^4 (4 h) and 4.14 × 10^4 (6 h). Label recovered in cytoplasmic fractions (in counts per minute) at 37 C was 9.2 × 10^4 (6 h); at 41 C, 2.54 × 10^4 (4 h) and 6.55 × 10^4 (6 h). (A) Label isolated from nuclei of cells 6 h postinfection at 37 C. (B and C) Label isolated from nuclei of cells 6 and 4 h, respectively, postinfection at 41 C. Symbols: Acid-insoluble tritium, O; density, ●.*

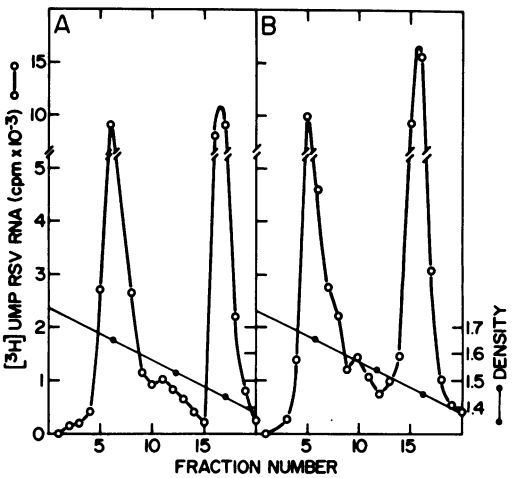

FIG. 4. *Equilibrium Cs_2SO_4-formaldehyde centrifugation of [^3H]uridine-labeled LA335 spontaneous revertant RNA isolated from nuclei of infected CEF. The experimental procedures were as described in Fig. 2, except that a spontaneous revertant (labeled with [^3H]uridine; titer, 3 × 10^4 FFU/ml at 37 C) was used to infect CEF cells. No difference in reverse transcriptase activity in LA335 revertant was detected as measured by deoxyadenylate-deoxythymidylate co-polymer synthesis after incubation at 37 and 41 C (20% inactivation at both temperatures after 120 min); multiplicity of infection was 0.074. Cell fractions were prepared 5 h after infection, and radioactivity recovered (in counts per minute) was as follows: in cytoplasmic fractions at 37 C, 4.3 × 10^5; at 41 C, 3.5 × 10^5; in nuclear fractions at 37 C, 1.72 × 10^5; at 41 C, 1.79 × 10^5. (A) Infection at 37 C. (B) Infection at 41 C. Symbols: Acid-insoluble tritium, O; density, ●.*

that the formation of single-stranded viral RNA-DNA covalent hybrid molecules requires active reverse transcriptase.

DISCUSSION

The observations presented above indicate that viral RNA covalently linked to DNA can be isolated from nuclei of infected CEF cells at a multiplicity of infection of 1 or less. This suggests that viral RNA can be transcribed to DNA in nuclear cell fractions. Dales and Hanafusa (4), on the basis of audioradiographic studies of RSV-infected CEF cells, have come to similar conclusions. In contrast, Varmus et al. (Cold Spring Harbor Symp. Quant. Biol., in press), using a heterologous cell system and high multiplicity of infection, reported the isolation of large double-stranded "proviral" DNA in the cytoplasm of B77-infected duck cells early after infection by hybridization techniques. Whether such a DNA intermediate is present in the cytoplasm of infected CEF cells is not known. We have so far been unable to detect the presence of large cytoplasmic viral-specific DNA in CEF labeled with [^{3}H]thymidine at the time of RSV infection. Furthermore, attempts to isolate viral RNA-DNA covalent hybrid in nuclei of B77-infected duck cells has also been unsuccessful.

The molecular weight of the largest RSV RNA-DNA covalent hybrid structures isolated from nuclei as measured in dimethyl sulfoxide (80%) sucrose gradients was approximately 10^6 to 2.5 × 10^6. Since hybrid structures contained on the average 50% DNA, this suggests that the molecular weight of the DNA in hybrid structures is greater than 10^5. This also suggests that the RNA primer in vivo may not be solely 4*S* RNA molecules. The DNA provirus in the B77-infected duck cells has a reported molecular weight of 3 × 10^6 (single-stranded) (Varmus et al., Cold Spring Harbor Symp. Quant. Biol., in press). Since purified reverse transcriptase is capable of synthesizing only small DNA pieces in vitro (6 to 7*S*) (7) covalently linked to

RNA, then an elongation factor(s) that permits reverse transcriptase to generate large DNA must be present in cells. Attempts to find reverse transcriptase elongation factors in avian myeloblast cells and Friend leukemia mouse cells are now in progress.

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