Fate of Viral RNA of Murine Leukemia Virus after Infection

(RNA tumor virus/RNA-DNA hybrid/Cs2SO4 centrifugation)

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ABSTRACT [3H]Uridine-labeled Rauscher leukemia virus was used to infect mouse embryo fibroblasts. After the infected cells were separated into nuclear and cytoplasmic fractions nucleic acid was extracted by sodium dodecyl sulfate–phenol–chloroform treatment and analyzed by Cs2SO4 and sucrose density gradient centrifugation. Between 45 and 70 min after infection a transient and synchronized shift of the acid-insoluble radioactive peak toward the RNA-DNA hybrid region occurred in both the nuclear and cytoplasmic fractions. The density of the cytoplasmic hybrid shifted to 1.56 g/ml (RNA = about 30%), while the sedimentation rate decreased from 36 S to 14 S; however, the density of the nuclear hybrid shifted to 1.58–1.68 g/ml (RNA = 57–17%, respectively), while its sedimentation rate remained about 65 S. The hybrids in both the nuclear and the cytoplasmic fractions still showed hybrid density after heat denaturation. The processes of the early stages of RNA tumor virus infection are discussed with regard to the functions of viral RNA-dependent DNA polymerase (reverse transcriptase) and a possible integration of viral genetic information into the host chromosome.

DNA synthesis in cells in the early stages of infection by an RNA tumor virus appears to play an important role in both the expression of transformation (1, 2) and the production of the progeny viruses (3). DNA copies homologous to the viral RNA have been found in virus-producing (4, 20) as well as virally transformed cell lines (5). These results suggest that reverse transcription of the viral information into DNA and the integration of this DNA into the host chromosome may be necessary for the maintenance and the expression of the viral information (6).

The process of the integration of the viral genetic information, however, is entirely obscure. In order to study these processes, we followed the fate of [3H]uridine-labeled parental RNA molecules of murine leukemia virus in the early stages of infection of mouse embryo fibroblasts. RNA-DNA hybrid molecules with density intermediate between double-stranded DNA and RNA in Cs2SO4 density centrifugation were detected. The hybrids found in the nuclear fractions had characteristic lower density and larger size than those found in the cytoplasmic fractions.

MATERIALS AND METHODS

Virus and Cell Lines. A cell line producing Rauscher leukemia virus (RLV), JLS-V9 (7), and mouse embryo fibroblasts (MEF) of BALB/c strain were used as a donor of the virus preparations, and as the host cells, respectively. Cells and virus were checked regularly and found free from contamination by mycoplasma. Both of the cell lines were obtained from Flow Laboratories, Inc., Rockville, Md.

Preparation of Virus. JLS-V9 cells in roller bottles were incubated for 2 days at 37°; the culture was in log phase and about 90% confluent. The medium was changed to 30 ml per bottle of minimum essential medium (MEM, Flow Laboratories) supplemented with 3% fetal bovine serum (FBS, Flow Laboratories) and 100 μCi/mI of (5,6-3H)uridine (46.2 Ci/mmole, New England Nuclear). After 10- to 14-hr incubation at 37°, the radioactive medium was discarded and an equal volume of fresh MEM containing 3% FBS was added to each bottle. The virus was collected for 90 min after the medium change. DNase I (Worthington Biochemical Corp.; RNase-free) was added to give a final concentration of 10 μg/ml for the last 30 min of each collection period. This collection procedure was repeated after another feeding with nonradioactive medium. The combined medium containing radioactive virus was purified by sucrose gradient centrifugation. Virus was pelleted onto a 60% sucrose cushion and then banded in a 15–50% sucrose gradient. The peak fraction of radioactivity in the final sucrose gradient (density = 1.16 g/ml) was used as a preparation of purified virus. The purified virus was diluted with 5 volumes of Earls’ balanced salt solution (BSS, Flow Laboratories) and used for infection on the same day the purification was done.

Infection with 3H-Labeled Viruses. Monolayers of MEF were plated 8–12 hr before infection at a density of 1.5 × 10⁶ cells per 60-mm plastic petri dish with 5 ml of MEM supplemented with 10% FBS, and incubated at 37°. These monolayers were treated with 12 μg/ml of DEAE-dextran at 37° for 30 min before infection. The infection was started by replacing the DEAE-dextran-containing medium with 0.5 ml of the diluted, purified 3H-labeled virus per plate. These viral preparations (0.5 ml) contained 6.3 to 9.5 × 10⁴ cpm of radioactivity, 1 to 5 × 10⁶ particles by electron microscope counting (8), and a tissue culture infecting dose of 10⁴ by XC cell assay (9). After 30-min incubation at 37°, 5 ml of prewarmed MEM containing 10% FBS was added to the plates. The adsorption of the radioactivity to these monolayers was about 14% under these conditions. Zero time was defined as the time of the addition of the virus preparation to each plate.

Fractionation of the Infected Cells. Eight minutes before each sampling time the medium of the plates was aspirated and 5 ml of prewarmed 0.25% trypsin in BSS was added to each plate.
After the incubation with trypsin at 37°, the monolayers were scraped with a rubber policeman and the cell suspensions were transferred to centrifuge tubes containing 5 ml of precooled MEM with 10% FBS. These cell suspensions were washed once with cold-phosphate-buffered saline (Flow Laboratories) and fractionated into cytoplasmic and nuclear fractions by treatment with a 1% solution of the neutral detergent NP-40 (Shell Laboratories) in 0.14 M NaCl, 0.02 M MgCl₂, and 0.02 M Tris·HCl, pH 7.2 [modified from Penman (10)]. The double detergent wash of the perinuclear membrane was omitted. The supernatants of the NP-40 treatment and the wash by 1% NP-40 were combined as a cytoplasmic fraction. The precipitate, called here the nuclear fraction, was suspended with 4 ml of 1% NP-40 solution.

**Extraction of Nucleic Acid.** Nucleic acids were extracted from both the cytoplasmic and nuclear fractions, as well as from the total infected cells without the fractionation. In the latter case, the monolayers of the infected cells were treated with trypsin and washed by the procedure described above. The suspension of the washed infected cells was lysed by the addition of 4 ml “lysis buffer” of 100 mM Tris·HCl, pH 7.2, 50 mM EDTA, 0.2 M NaCl, and 2% sodium dodecyl sulfate (Bio-Rad Laboratories) per plate. After 5-min incubation at room temperature, the nucleic acids were extracted from the lysate with phenol by the procedure described below.

Immediately after the fractionation, EDTA was added to the cytoplasmic and nuclear fractions to a final concentration of 40 mM and an equal volume of “lysis buffer” was added. The nucleic acids were extracted from these sodium-dodecyl-sulfate-treated cytoplasmic and nuclear fractions by the addition of an equal volume of 80% phenol in 0.5 M Tris·HCl, pH 7.2, and by gentle rotation for 20 min at room temperature. The concentration of DNA in the samples was less than 0.75 μg/ml in the nuclear fraction. An equal volume of chloroform–isoamyl alcohol (24:1) was added and the mixtures were gently rotated for another 10 min. After the separation of the aqueous phase by centrifugation, the residual phenol was extracted by gentle mixing with water-saturated ether. Ether in these nucleic acid preparations was evaporated with nitrogen gas at room temperature. The nucleic acid preparations were diluted to 9 ml with sterile buffer (10 mM Tris·HCl; 10 mM EDTA, pH 7.2). One-milliliter and 8-ml portions of these preparations were analyzed for their molecular size and density distribution of the radioactive components, by sucrose and Cs₂SO₄ gradient centrifugations, respectively.

**Preparation of P-Labeled RNA Markers.** P-Labeled viral RNA (70 S) of JLS-V9 was a gift of Dr. N. Tsuchida (11). P-Labeled ribosomal RNA, 18 S and 28 S, used as the marker in the sedimentation analysis, was prepared from a culture of JLS-V9 in MEM containing 2% FBS and 100 μCi/ml [P]-phosphate (carrier-free, New England Nuclear) followed by phenol extraction of the cytoplasmic fraction after separation by the method described above.

**Sucrose Gradient Centrifugation.** A one-ml solution of the nucleic acids from whole infected cells, nuclei, or cytoplasm was added to 10–20 μl of P-labeled RNA (about 1000 cpm) and P-labeled 70 S viral RNA (450 cpm). These samples were layered on 34 ml of 10–30% sucrose gradient solution in 50 mM Tris·HCl, 0.1 M NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate, pH 7.2. The content of DNA of the nuclear fractions in sucrose gradient was less than 20 mg/ml. For the nuclear fractions the gradients were centrifuged at 17,500 rpm for 8.5 hr, and for the cytoplasmic fractions, at 25,000 rpm for 11 hr at 20° in a Spincro SW27 rotor. After centrifugation, 1.6-ml fractions were collected from the bottom of the tubes. Half a milliliter of each fraction was precipitated with 10% trichloroacetic acid after the addition of 100 μg of carrier calf thymus DNA and the radioactivity of the precipitate was determined.

Cs₂SO₄ Gradient Centrifugation. One hundred fifty-four g of Cs₂SO₄ (Harshaw Chemical) was dissolved in 100 ml of distilled H₂O. The solution was filtered through Millipore HA filters and autoclaved at 120° for 15 min. Eight-milliliter portions of the nucleic acid preparations were each added to 9 ml of Cs₂SO₄ solution. Centrifugation was in polycarbonate tubes with 1 ml of paraffin oil, for 65 hr at 28,000 rpm and 20°, in a Spincro rotor 30. DNA concentration was 34 μg/ml. All gradients were collected from the bottom of the tubes, with 0.8 ml per fraction. Portions (0.2-ml) of each fraction were precipitated with 10% CH₃COOH and 100 μg of carrier calf thymus DNA and the radioactivity was determined. Another 0.2-ml portion of each fraction was treated with 0.5 M NaOH at 60° for 45 min, and the CH₃COOH-H-insoluble radioactivity was determined.

**Heat Denaturation of RNA-DNA Hybrid Molecules.** Fractions of Cs₂SO₄ gradient centrifugations, indicated in the Results, were pooled and dialyzed against buffer containing 15 mM NaCl, 1.5 mM sodium citrate, and 10 mM EDTA, pH 7.2, at 4° overnight. The dialyzed samples were heated at 95° for 6 min and then rapidly cooled in ice. These heat-denatured samples were adjusted to volume and mixed with Cs₂SO₄ solution. Centrifugation was for 65 hr at 38,000 rpm and 20°, in a Spincro 40 rotor. The acid-insoluble radioactivity of each fraction was determined.

**RESULTS**

**RNA-DNA Hybrids in Cells Infected with H-Labeled RLV.** Purified [H]uridine-labeled RLV was used to infect MEF. The nucleic acids were extracted from the infected cells at various times after infection. The density distribution of the radioactive components of these nucleic acid preparations was analyzed by Cs₂SO₄ density centrifugation (Fig. 1). Most radioactivity from the infected H-labeled virus was not recovered from the dodecyl sulfate–phenol extract immediately after the infection (Fig. 1a). This loss of radioactivity presumably resulted because the trypsinization immediately after infection washed all the adsorbed H-labeled virus out of the cells. When one-tenth of the infecting dose of labeled virus was added to a noninfected cell suspension of MEF immediately before the extraction of the nucleic acids, the radioactivity was recovered as a single peak at the density of 1.70 in the Cs₂SO₄ centrifugation (Fig. 1b, c, and d). In samples taken at 45, 60, and 70 min after infection, the radioactivity was distributed in various peaks of intermediate density between DNA (the density of MEF DNA was 1.42) and viral RNA (the density of the viral RNA was 1.70). The density of these peaks seemed to shift from 1.70 to 1.485 as the sampling time proceeded from 45 to 70 min (Fig. 1b, c, and d). The radioactivity of the extracted nucleic acids, however, returned to the original position of viral RNA at 90 min after the infection (Fig. 1c). Similar profiles were obtained and no further shift of the radioactive peaks was observed at 120, 150, and 180 min after infec-
FIG. 1. CsSO₄ Centrifugation of nucleic acids from cells infected by [3H]uridine-labeled RLV. From (a) to (e), the nucleic acids were extracted from the infected cells without the cellular fractionation, at the indicated time after infection. Those of the cytoplasmic and nuclear fractions sampled at the indicated time after infection are shown in (f), (g), and (h), and those of the nuclear fractions in (i), (j), and (k), respectively. An uninfected MEF culture was harvested as described in Methods and one-tenth of the infecting dose of [3H]labelled RLV was added immediately before the extraction of the nucleic acids and analyzed in CsSO₄ centrifugation (l). Black circles show acid-insoluble radioactivity (and density) and white circles show radioactivity after alkaline digestion. The numbers indicated at each peak show the densities of the peaks.

Localization of RNA-DNA Hybrids in the Infected Cells. In order to localize these RNA-DNA hybrid molecules in the infected cells, the infection of MEF by RLV was repeated and the trypsinized suspension of the infected cells was fractionated into nuclear and cytoplasmic fractions. The nucleic acid extracts were also analyzed by CsSO₄ centrifugation (Fig. 1f to k). At 45 and 60 min after infection most of the radioactivity was found in the cytoplasmic fractions, and the profiles were similar to those of the uninfected cells at the same time points, shown in Fig. 1b and c. At 70 min, however, the nuclear fraction contained one predominant peak, at a density of 1.48 g/ml, which did not exist in the profile of the cytoplasmic fraction at any time point.

Sedimentation Rate of RNA-DNA Hybrid Molecules. The molecular size of these hybrid molecules was determined. The same samples of the nucleic acids that were used in the experiments in Fig. 1f through k were analyzed on sucrose gradient centrifugation. The results are shown in Fig. 2. The [3H]radioactivity of the nucleic acids from the cytoplasmic fractions sedimented in three different peaks; 36 S, 21 S, and 14 S (Fig. 2a, b, and c). As time proceeded after infection, the [3H]radioactivity in the 36S component decreased, while that in the 14S components increased. This suggests that the infecting viral RNA is degraded in the cytoplasm after infection. Most of the [3H]radioactivity of the nuclear fraction, however, sedimented in one peak at about 65 S (Fig. 2d, e, and f).

In order to determine the correlation between the peaks in CsSO₄ centrifugation and those in the sucrose gradient analysis, aliquots of the fractions of the sucrose gradient were pooled as shown by the roman numerals I and II in Fig. 2f and b, and precipitated by ethanol. These samples were analyzed by CsSO₄ centrifugation. The fast sedimenting fraction in sucrose gradient analysis, the pool I of Fig. 2f, showed a predominant peak at the density of 1.49 g/ml in the CsSO₄ centrifugation, although two minor peaks appeared at densities of 1.73 and 1.62. On the other hand, the pool III (Fig. 2b) gave three peaks at densities of 1.72, 1.61, and 1.57. The peak at a density of 1.57 is similar to the density of the predominant peak in the cytoplasmic fraction in Fig. 1g. From these results it is apparent that the nuclear predominant peak at a density of 1.48 and the cytoplasmic predominant peak at a density of 1.55 correspond to the peaks of 65 S and 21 S in the sucrose gradient in Fig. 2f and b, respectively.

Covalent Binding of Viral RNA and DNA in the Hybrids. We investigated whether or not these DNA-RNA hybrid molecules contain covalent bonds between the DNA and RNA molecular species. Portions of the peak fraction in CsSO₄ centrifugation (Fig. 1h and k) were dialyzed against
a low-salt buffer, heated to 95° for 6 min, and then rapidly cooled in ice. These heat-denatured samples were again applied to Cs2SO4 density centrifugation. The hybrid molecules found in the cytoplasmic fraction, whose original density was 1.55, separated into two peaks at densities of 1.59 and 1.48. A small portion of the radioactivity was still present at the original density. The hybrid molecules found in the nuclear fraction, whose original density was 1.48, showed two separate peaks at densities of 1.53 and 1.42. It is concluded that both of the hybrids in the cytoplasmic and nuclear fractions have covalent bindings between radioactive RNA and DNA.

Inhibition of Hybrid Formation by Arabinocytidine. The effect of an inhibitor of DNA synthesis was studied on the formation of these hybrid molecules. Arabinocytidine (aCyd, Parke, Davis, & Co.; frequently called cytosine arabinoside) was added to the media of one of the monolayers of MEF at a concentration of 50 μg/ml from 30 min before, to 60 min after, the viral infection. At 70 min after infection the trypsinized cells were fractionated and the nucleic acids were extracted from the cytoplasmic and nuclear fractions. Cs2SO4 gradient centrifugation of the nucleic acid preparations was performed. The results are shown in Fig. 3. The radioactivity of the cytoplasmic fraction of the aCyd-treated cells peaked mainly at a density of 1.70 g/ml, which is that of the original viral RNA (Fig. 3c) and no detectable peaks were found in the nuclear fraction (Fig. 3d), while the control experiment of the nontreated, infected cells showed the typical peaks of the hybrids at densities of 1.55 and 1.45 in the cytoplasmic and nuclear fractions, respectively (Fig. 3a and b). Sucrose gradient analyses of the cytoplasmic fraction of these aCyd-treated and untreated samples were also carried out. The radioactivity of the aCyd-treated cells sedimented in a peak at 70 S, while those of the untreated, infected cells showed a typical sedimentation pattern similar to the profiles shown in Fig. 2c and f (results not shown). It is concluded that aCyd inhibits the formation of the hybrids in both the cytoplasmic and nuclear fractions and conserves the infecting viral RNA at its original density and sedimentation rate.

**DISCUSSION**

Prelabelled MLV virus RNA was recovered in the region of hybrid density in the region of density by Cs2SO4 density centrifugation within 45-70 min of infection of MEF. The shift of the density of the radioactive viral RNA is very synchronous and transient. More than 95% of the recovered radioactivity was alkali-sensitive. These radioactive hybrid molecules are not the artifacts of the extraction of the nucleic acids and Cs2SO4 density centrifugation, because the preparation from a mixture of the uninfected MEF and the [3H]uridine-labeled RLV showed a single sharp peak of radioactivity at a density of RNA (1.70 g/ml) (Fig. 1f). Another control finding is the inhibition of the formation of these hybrid molecules by aCyd. aCyd is known as an inhibitor of DNA synthesis that inhibits only the RNA-dependent DNA polymerase of RNA tumor virus (12), but also the DNA synthesis of the host cell (13). The focus formation of the sarcoma-virus-infected cells is also inhibited (3). Therefore, it may be suggested that the viral RNA-dependent DNA polymerase and/or a similar enzyme(s) of the host cell is involved in the processes of the formation of these hybrid molecules.

The differences between the hybrid molecules in the cytoplasmic and those in the nuclear fraction are very evident in their densities and sedimentation profiles. The hybrid molecules in the cytoplasmic fraction have density up to 1.55 (corresponding to a RNA/DNA ratio of about 1/1), while those in the nuclear fraction have density as high as 1.48 (RNA/DNA ratio 1/4). The sedimentation rates of the radioactive components of the cytoplasmic fraction are much smaller than those of the nuclear fraction, and the amounts of the lighter components increase as the infection proceeds, while those of the nuclear fraction remain constant at 65 S through 70 min after infection. The mass of the intact RLV RNA (70 S) is about 12 × 10⁶ daltons (14). Therefore, the hybrid molecules of the nucleus may be large enough to contain the 70S viral RNA. From the density of the heat-denatured hybrids the RNA content of the heavier strand of the nuclear hybrid is about 40%. It is suggested that the whole genome of the infected virus might be conserved on one strand of the hybrid molecules in the nuclear fraction. Alternatively, the RNA portion might be integrated in the numerous sites of the hybrids as many small pieces, and the length of each RNA piece might become smaller as the strands of the DNA copies extend and replace them as the infection proceeds.

The radioactive components of the nucleic acids from the cells infected by [3H]labeled RLV returned to the density of 1.70 after 90 min of the infection. The radioactivity was mostly found in the cytoplasmic fractions at this time of infection (the results are not shown in figures). This might be explained as follows: the radioactive RNA strands might be cleaved out and released from the hybrid molecules by an unknown mechanism of the infected cells, or alternatively, radioactive ribonucleotides, which may be released from the hybrid molecules by a RNase H (15, 16) of the infected cells, may be incorporated into the cellular RNA fraction. Both of these processes might coincidentally occur in the infected
cells. It should be recognized that the RNA molecules analyzed in these experiments may not be representative of the biologically critical viral RNA molecules, because as few as 10^-4 of the RNA molecules were in particles that yielded plaques of the XC-cell assay (9). These results are in conflict with those of Varmus et al. (20) who reported that the amount of viral DNA formed after infection is approximately equal to the number of infectious virions in a preparation (not the number of total virions as indicated by the present results). Different techniques of assaying viral DNA might be responsible for the different findings.

Rokutanda et al. (17) and Bishop et al. (18) analyzed the density of the template RNA after the in vivo reaction with the viral DNA polymerases of murine sarcoma virus and Rous sarcoma virus, respectively. They reported that the densities of the template RNA did not change in either of the cases. This apparent discrepancy between the in vitro polymerase reaction and the in vivo fate of the infecting viral RNA might be explained as follows: the size of the products of the in vitro DNA polymerase is less than 10 S (17), and the association of the lesser amount of these small DNA pieces with a larger amount of the template RNA may not affect the original density of the templates (19), while the in vivo transcription occurs on the entire genome of the infecting virus, to the extent that the complex of the template RNA and the DNA copies shows the hybrid density.

The present communication deals with none of the biological functions of these RNA-DNA hybrid molecules. We can, however, simply assume that first in the cytoplasm the hybrid (1.56 g/ml) might be made in a large excess, and a considerable portion of these cytoplasmic hybrids might be translocated into the nuclei. The hybrids in the cytoplasm that are not incorporated into the nuclei might be degraded quickly. The translocated RNA-DNA hybrid molecules in the nuclear fraction may be the intermediates of the synthesis of the double-stranded DNA copies of the viral genome. These double-stranded DNA copies of the viral genome may serve to integrate the viral information into the host chromosome (6). Alternatively, these RNA-DNA hybrid molecules may possibly be integrated into the host chromosome directly. The latter hypothesis suggests that the hybrid molecules in the nuclear fraction are an intermediate of these integration processes, i.e., they might be covalent complexes of the host DNA pieces and the viral RNA-DNA hybrid synthesized by a reverse transcriptase of the virions or the host cells. More critical experiments have been done by the hybridization of these hybrids with the viral DNA probe and the host DNA rat cells that carry few detectable sequences homologous to those of the mouse leukemia virus (22). J. Leis and J. Hurwitz (personal communication) have recently found similar RNA-DNA hybrid molecules in the nuclear fraction of the cells infected with avian leukosis viruses, and Sveda et al. (21) have reported hybrid molecules in cells infected by murine viruses. Their results are consistent with those reported here.

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