Reverse Transcription of the Viral Genome Associated with the Plasma Membrane after Infection with RNA Tumor Viruses

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SUMMARY

Electron microscopic autoradiography of 3T3/BALB and chicken embryo cells infected with murine sarcoma virus or Rous sarcoma virus (RSV) showed thymidine-3H incorporation in the region of the plasma membrane beginning 1 hr after infection. The photopositive grains dispersed diffusely in the cytoplasm as the postinfection time was prolonged. Reverse transcriptase activity generated by virus infection was assayed for by incubating isolated cell organelles in a transcription mixture with labeled deoxythymidine 5'-triphosphate. The DNA products were hybridized with viral RNA and then analyzed by isopycnic centrifugation in Cs2SO4 to determine whether any sequence homology existed. The plasma membrane showed the highest activity. DNA hybridizable to viral RNA was also isolated from the plasma membrane fraction of RSV-infected cells. These results indicated that the initial DNA copy of viral RNA is synthesized at the plasma membrane about 1 hr after infection and this synthesis continued for up to 7 to 10 hr. The DNA synthesized in vitro with isolated plasma membranes was found to consist of small homogeneous fragments having a molecular weight of about 1.5 x 10^6. In contrast, the virus-specific DNA synthesized in the living cells and extracted from the plasma membrane of RSV-infected chicken embryo cells was heterogeneous in size ranging from 10^5 to higher than 10^6 daltons. No evidence was found for virus-specific, reverse transcription associated with plasma membranes in uninfected cells. Neither microsomes, ribosomes, nor mitochondria were found to be involved in the initial transcription of viral RNA a short time after infection.

INTRODUCTION

The discovery of a RNA-dependent DNA polymerase in RNA tumor viruses (3, 13) provided evidence that viral information could be transmitted through specific DNA synthesized by the viral enzyme. However, the mode of replication and the nature of the DNA products in the virus-infected cell are still largely unknown.

A previous autoradiographic study showed that an unusual TdR-3H incorporation occurred in the cytoplasm of 3T3/BALB cells a few hr after MSV infection. No such cytoplasmic DNA synthesis was found in uninfected cells or cells incubated with UV-inactivated virus (8). A study by Dales and Hanafusa (4) suggested that virion-directed DNA transcription and replication occurred in the nuclei of CE cells infected with avian RNA tumor viruses. Bader (1) and Bader and Bader (2) have published biochemical and biologic evidence that the replicative intermediate DNA of the RNA tumor virus genome was synthesized as early as 1 hr after infection.

In order to elucidate this obviously complex sequence of intracellular events, 3 experimental approaches were used in these studies to determine the intracellular localization of the viral RNA template and its associated reverse transcriptase.

MATERIALS AND METHODS

Cells and Viruses. 3T3/BALB cells were plated in MEM containing 10% fetal bovine serum. After an additional 24 hr the medium was removed and each dish (6.3 x 10^6 cells per 25-cm plastic culture dish; 1 dish for autoradiographic studies and 10 dishes for biochemical studies in each experimental group) received either a Harvey or Moloney strain of MSV in 5% serum and after which it was incubated at 37°C for different times as described in the text. The multiplicity of infection was 200 (30,000 particles per cell). Secondary cultures of CE cells grown in the MEM with 10% serum and tryptose phosphate broth were similarly infected with Bryan "high-titer" strain of RSV (3,200 particles per cell) and incubated at 38°C for different times. Chronically transformed cells were produced by subculturing the infected cells for more than 40 days. The virus preparations were tested for bacterial and mycoplasma contamination before infection. Control cells were either uninfected or incubated with UV-inactivated virus (60 ergs/sq mm for 1.5 hr). All of the virus-infected cells were washed extensively with Earle's balanced salt solution before labeling with radioactive compound or isolation of subcellular organelles.

Purification of Viral RNA. Virus-producing cells (10 plates) were washed and incubated with MEM containing 5% serum and uridine-3H (17.4 Ci/mmol, 50 μCi/ml) for 12 hr. The media were harvested during this period at 4-hr intervals. The further purification of the virus and the isolation of 60 to 70 S viral RNA has been described elsewhere (9). The specific activity of the purified viral RNA was 7 x 10^4 μCi/μg.

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The abbreviations used are: TdR-3H, thymidine-3H; MSV, murine sarcoma virus; CE, chick embryo; MEM, Eagle's minimal essential medium; RSV, Rous sarcoma virus.
Autoradiography. The method used for electron microscopic autoradiography has been described previously (8). The cells infected with viruses as described above were incubated for 1 hr and washed with Earle's balanced salt solution, and then MEM containing 5% serum was added. Each dish received TdR-3H (14.1 Ci/mmmole, 125 µCi/ml) at different times and incubated as specified in the figure legends. The samples were then fixed by treatment for 1 hr with 2.5% glutaraldehyde in s-collidine buffer (pH 7.4) followed by treatment for 1 hr with 1% osmic acid. Each sample was then treated with cold 5% trichloroacetic acid for 30 min, washed, and dehydrated with acetone; wash solutions were monitored for release of radioactivity. By the final wash, no soluble radioactivity was recovered. After embedding the specimen in an Epon-Araldite mixture, the samples were incubated in 55, 45, and 40% sucrose solutions and centrifuged at 90,000 g for 2 hr (all of the sucrose solutions contained 5 mM MgCl₂). The membrane collected from the 45% sucrose solution, which in turn was on top of a 50% sucrose solution, and layered on top of a 30% sucrose solution, was further described in the figure legends and washed extensively with 20% sucrose solution, and then MEM containing 5% serum was added. Isopyknic centrifugation in Cs₂SO₄ was carried out by resuspending a portion of the isolated nucleic acids in a Cs₂SO₄ solution to yield a starting density of 1.55 g/ml followed by centrifugation for 60 hr at 20° and 45,000 rpm in an IEC A-321 rotor. Fractions were collected and the radioactivity present in acid-precipitable material was collected on glass fiber filter papers and assayed.

Alkaline Sucrose Gradient Fractionation of the Product of in Vitro DNA Synthesis. To analyze the ³²P-labeled DNA products obtained as described above, the ethanol-precipitated nucleic acids were mixed with 0.4 ml of 0.6 N NaOH and incubated for 2 hr at 37°. Two-tenths of a ml of this mixture was placed on top of a 4 to 20% linear sucrose gradient which contained 0.9 M NaCl, 0.1 N:0.3 N NaOH, and 5 mM disodium EDTA and was then centrifuged for 8 hr at 20° and 50,000 rpm in Spinco SW 65 rotor. Sedimentation coefficients and apparent molecular weights were determined as described by Dingman (5).

DNA-RNA Hybridization. The remaining 0.2 ml of the dTMP-³²P-labeled denatured DNA products described above were neutralized with 1 N HCl. An equal volume of uridine-³H-labeled 60 to 70 S viral RNA (90 µg; 2100 cpm/10 µl) was added and the mixture was brought to 0.33 M NaCl and boiled at 100° for 1 min. After quick cooling of the solution in ice, an equal volume of formamide was added and the mixture incubated for 20 hr at 37°. To this was added marker rRNA (Escherichia coli 16 S and 23 S) and calf thymus DNA and then saturated Cs₂SO₄ to yield a starting density of 1.55 g/ml. The mixture was then subjected to isopyknic centrifugation. After fractionation the density and optical adsorption (A₂₆₀) of each fraction was determined. The marker RNA was tested and proved not to hybridize with dTMP-³²P-labeled virus-specific DNA.

Isolation of Virus-directed Newly Synthesized DNA from Membranes of RSV-infected CE Cells. CE cells (6.3 x 10⁸) were infected with RSV (3.2 x 10⁸ particles per cell) for 3 hr in the presence of 50 µCi of TdR-³H per ml (17.3 Ci/mmmole) and washed. The plasma membrane was isolated (Tris-MgCl₂ method), total nucleic acids were extracted, precipitated in ethanol, and then dissolved in 0.2 ml of 0.6 N NaOH to denature DNA and eliminate RNA as described above for the in vitro DNA products. The denatured DNA was then hybridized with unlabeled to 60 to 70 S RSV-RNA (180 µg in 0.5 ml of 0.01 M Tris buffer, pH 7.4, with 1 mM EDTA) and fractionated by isopyknic centrifugation in Cs₂SO₄ as described earlier. An aliquot of the fractions having a hybrid density (1.575 to 1.615 g/ml in Chart 5) was either (a) analyzed by isopyknic centrifugation in alkaline Cs₂SO₄ (Chart 6); (b) dialyzed against 0.01 M NaCl with 4 mM disodium EDTA, pH 7.8, the nucleic acids reprecipitated in ethanol, denatured in alkaline again, and rehybridized with ³²P-labeled to 60 to 70 S RSV-RNA (prepared the same way as for RSV-RNA-³H except that 50 µg of protein in the form of subcellular particles; and 0.1% of Triton X-100. The reaction was stopped by cooling in ice and by the addition of disodium EDTA to 1 mM followed by sodium dodecyl sulfate to 1%. The nucleic acids were extracted with phenol and precipitated with ethanol. Isopyknic centrifugation in Cs₂SO₄ was established by resuspending a portion of the isolated nucleic acids in a Cs₂SO₄ solution to yield a starting density of 1.55 g/ml followed by centrifugation for 60 hr at 20° and 45,000 rpm in an IEC A-321 rotor. Fractions were collected and the radioactivity present in acid-precipitable material was collected on glass fiber filter papers and assayed.

Isolation of Plasma Membrane (Tris-MgCl₂ Method). A method described by Warren et al. (14) was used with minor modifications. The 3T3/BALB or CE cells (6.3 x 10⁸) were infected with virus and incubated for different times as described in the figure legends and washed extensively with 50 mM Tris-chloride buffer, pH 7.4, containing 5 mM MgSO₄ and 0.1 M NaCl. Electron microscopic autoradiography was carried out as described above.

Isolation of Other Subcellular Organelles. The isolated subcellular organelles were subjected to isopyknic centrifugation. After fractionation the density and optical adsorption (A₂₆₀) of each fraction was determined. The marker RNA was tested and proved not to hybridize with dTMP-³²P-labeled virus-specific DNA.

DNA Transcription in Vitro. The isolated subcellular organelles were incubated for 45 min at 37° with a transcription mixture (3) which contained in 3.0 ml: 150 µmoles of Tris-chloride, pH 8.3; 18 µmoles of magnesium acetate; 180 µmoles of NaCl; 60 µmoles of dithiothreitol; 2.4 µmoles each of dATP, dCTP, dGTP; 0.01 µmole of dTTP-³²P (15 Ci/mmmole, International Chemical and Nu-
Plasma Membrane-bound Reverse Transcription of Viral RNA

RESULTS

Electron Microscopic Autoradiography. Results obtained from light microscopic autoradiography have been described earlier (8). Electron microscopic autoradiography done in this present study indicated that photopositive grains were closely associated with the plasma membrane or peripheral portions of the cytoplasmic processes of 3T3/BALB cells infected with MSV for 1 hr followed by pulse labeling for 1 to 2 hr as shown in Figs. 1 to 3. The number of grains was, however, not more than 5 per cell in most of the labeled cells. The cytoplasmic labeling became more evident when the cells were labeled between 6 and 10 hr after infection and plasma membrane labeling was then diminished (Fig. 4). Very little mitochondrial labeling was found in either the virus-infected or the uninfected cells. All of the extranuclear labeling described above disappeared after treatment of the cells with DNase. No bacterial and mycoplasmic contamination could be detected by electron microscopy. In another control experiment the 3T3/BALB cells and HeLa cells treated similarly for 4 to 10 hr with RSV did not show any cytoplasmic labeling.

In general, the autoradiographic findings obtained with the RSV system were not different from that of the MSV system described above.

In Vitro DNA Synthesis Using Isolated Subcellular Organelles. If the viral RNA genome and the viral-induced enzymes for reverse transcription are introduced into a cell by infection, then the initiation and/or elongation of a polydeoxyribonucleotide chain might proceed even after the cellular components were isolated and incubated under appropriate experimental conditions. In order to detect such potential in vitro reverse transcriptase activity specific for the viral RNA present in various subcellular fractions, 3T3/BALB and CE cells were infected with MSV and RSV, respectively. Subcellular organelles were then isolated at different times after infection and incubated in vitro with the transcription mix (see "Materials and Methods"). The isolated organelles were monitored by electron microscopy and proved to be free of intact virions (Fig. 5). Following incubation, the total nucleic acids were extracted with sodium dodecyl sulfate and phenol and analyzed by isopycnic centrifugation in Cs₂SO₄. The result is shown in Chart 1 where it can be seen that incorporation with membranes from MSV-infected cells results in the appearance of a dTMP-³²P-labeled band in a hybrid density region. Pretreatment of samples with RNase (10 µg/ml, in 0.1 M Tris-chloride buffer, pH 7.4, with 0.1 M NaCl, for 20 min), omission of 1 of the deoxynucleoside triphosphates, or omission of Mg⁺⁺ strongly inhibited the reaction.

Characterization DNA Products Synthesized in Vitro Using Isolated Plasma Membranes

RNA-DNA Hybridization. dTMP-³²P-labeled DNA products synthesized in vitro as described above were denatured in alkali and hybridized back to the original 60 to 70 S ³H-labeled viral RNA. In the first 2 hr after infection the isolated plasma membrane of CE exhibited a small but detectable amount of ability to support DNA synthesis complimentary to RSV-RNA. Three hr after infection the activity was markedly enhanced, particularly in the plasma membrane of MSV-infected 3T3/BALB cells (Chart 2). This in vitro DNA-synthesizing potential which was found in the plasma membranes of CE infected with RSV was maintained for up to 7 hr (Chart 3). The activity dropped significantly 24 hr after infection. It is conceivable that the viral RNA introduced in the cell may be transcribed partially or completely a short time after infection prior to the isolation of the plasma membrane as discussed in a later section. This early viral DNA synthesized in vivo (unlabeled) would compete with ³²P-labeled DNA synthesized in vitro in hybridization with ³H-labeled viral RNA resulting in different patterns of sedimentation behavior; shifting the complex structure into the hybrid region of Cs₂SO₄ fractions. The plasma membrane isolated from CE cells chronically transformed by RSV also showed ability to support the synthesis of DNA hybridizable to viral RNA.

Alkaline Sucrose Gradient Fractionation of in Vitro Synthesized DNA. In order to estimate the molecular

μCi of ³²P per ml were used in place of uridine-³H) (Chart 7); or (c) dialyzed as described above and analyzed in 4 to 20% alkaline sucrose gradients (Chart 8).

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infection, an attempt was made to isolate the virus-specific DNA from virus-infected cells. The presumptive viral DNA was labeled by incubating cells with TdR-3H for 3 hr starting immediately after infection with RSV. The plasma membrane was isolated, and the nucleic acids were extracted and treated with alkali to eliminate RNA. The resulting nucleic acid solution was then neutralized and hybridized with unlabeled viral RNA. This was fractionated by isopyknic centrifugation in neutral Cs2SO4. The band containing hybrid molecules (DNA-3H complementary to viral RNA, Fractions 11 to 15 of Chart 5) was (a) analyzed by isopyknic centrifugation in alkaline Cs2SO4, (b) denatured again in alkaline and rehybridized with 32P-labeled viral RNA, and (c) analyzed by zone sedimentation in an alkaline sucrose gradient (see "Materials and Methods"). The results indicated that the virus-specific DNA isolated from cells in this manner banded in the region expected for single-stranded DNA in alkaline Cs2SO4 (Chart 6). Practically all of the DNA-3H hybridized back again with 32P-labeled viral RNA (Chart 7). Finally, the virus-specific DNA synthesized in vivo was found to be heterogeneous in size ranging from 10^6 to larger than 10^8 daltons (Chart 8).

Reverse Transcription Activity Associated with Other Subcellular Organelles

The mitochondria and microsomes isolated from cells 3 hr after infection and from chronically transformed cells by RSV or MSV were tested for their ability to support in vitro synthesis of DNA homologous to the viral RNA. Only minor and inconsistent activity was found in the microsomes of newly infected CE cells which may be attributed to plasma membranes contaminating the microsome fraction. Virtually no activity was found in the mitochondrial fraction.

Characterization of Virus-specific DNA Synthesized in Vivo in the Plasma Membrane

Based upon the experimental results described above which indicated that the viral RNA might be transcribed into DNA at the plasma membrane a short time after infection, an attempt was made to isolate the virus-specific DNA from virus-infected cells. The presumptive viral DNA was labeled by incubating cells with TdR-3H for 3 hr starting immediately after infection with RSV. The plasma membrane was isolated, and the nucleic acids were extracted and treated with alkali to eliminate RNA. The resulting nucleic acid solution was then neutralized and hybridized with unlabeled viral RNA. This was fractionated by isopyknic centrifugation in neutral Cs2SO4. The band containing hybrid molecules (DNA-3H complementary to viral RNA, Fractions 11 to 15 of Chart 5) was (a) analyzed by isopyknic centrifugation in alkaline Cs2SO4, (b) denatured again in alkaline and rehybridized with 32P-labeled viral RNA, and (c) analyzed by zone sedimentation in an alkaline sucrose gradient (see "Materials and Methods"). The results indicated that the virus-specific DNA isolated from cells in this manner banded in the region expected for single-stranded DNA in alkaline Cs2SO4 (Chart 6). Practically all of the DNA-3H hybridized back again with 32P-labeled viral RNA (Chart 7). Finally, the virus-specific DNA synthesized in vivo was found to be heterogeneous in size ranging from 10^6 to larger than 10^8 daltons (Chart 8).
DNA which specifically hybridizes with viral RNA was estimated to be 0.24 pmole/μg of membrane protein added to the transcription mixture. By comparison, 0.22 pmole of TMP incorporation per μg of virion protein occurred when purified virions were used in place of the membrane for DNA transcription in vitro under condition similar to those we used. If only intact or partially damaged virions contaminating the isolated plasma membranes were responsible for the DNA-synthesizing activity found in vitro, then the isolated plasma membranes must have consisted of nothing but virions. Electron micrographs of the membrane fractions proved this was not the case as no virions were identified. This activity remained for an additional 7 hr. However, as shown by autoradiography, the incorporation of TdR-3H into the plasma membrane region of an individual cell was not changed or decreased at later times suggesting that the enhanced in vitro activity associated with the plasma membrane was a result of either an increased number of cells involved in the virus infection or the consequence of a lack of in vivo transcription of viral RNA at later stages of infection. The time of infection relative to the cell cycle (1) and infection with virions of varying activity could determine, in part, the time difference between the integration of the viral genome into the cell and its subsequent transcription.

The data obtained from the present experiments indicated a relatively low ability of other subcellular organelles of newly infected cells to support DNA synthesis as detected.

**DISCUSSION**

The present experiments were directed toward the question of the sequence and subcellular localization of the events which occur following the introduction of a viral RNA genome into a susceptible cell. In vitro DNA synthesis using isolated subcellular organelles was found to be a sensitive assay for the presence of viral RNA template and the enzymes involved in reverse transcription, while high-resolution autoradiography was useful for localizing the DNA products in situ at the subcellular level. The photopositive grains were observed at the plasma membrane region of virus-infected cells as early as 1 hr after infection and continued to increase for 3 hr. The results from in vitro assay using isolated plasma membranes also indicated that viral RNA and functioning enzyme are already present at this stage of infection. The incorporation of TMP-32P into DNA products synthesized in vitro using isolated plasma membranes from RSV-infected CE cells (Chart 4) and in vitro DNA products synthesized with purified RSV (7).

**Chart 4.** The dTMP-32P-labeled DNA produced in vitro by plasma membranes of CE cells infected with RSV (an aliquot of the same sample used for the experiment shown in Chart 3) was denatured in 0.6 M NaOH and analyzed by alkaline sucrose gradient centrifugation. The scale of apparent molecular weights was obtained from the measured sedimentation coefficients (5) using Studier’s equation (12).

This was in sharp contrast to the DNA product synthesized in vitro using isolated plasma membranes from RSV-infected CE cells (Chart 4) and in vitro DNA products synthesized with purified RSV (7).
by our in vitro transcription system. Therefore, the photo-positive grains found in the cytoplasmic regions at later stages of infection should probably be regarded as DNA synthesized previously at the plasma membrane and then transferred into these regions or as a result of DNA-dependent DNA synthesis. The activity for in vitro DNA synthesis found in transformed plasma membranes may be attributed to progeny virions, containing viral RNA and enzyme, budding out from the membrane, rather than the continued activity for viral DNA synthesis at this site stimulated by the original infecting viral genome. This concept is supported by the electron microscopic autoradiography of virally transformed cells in which TdR-$^3$H incorporation into plasma membrane region is low relative to newly infected cells.

The TdR-$^3$H-labeled virus-specific DNA extracted from plasma membranes of virus-infected cells gave more direct evidence for the presence of intracellular reverse transcription. It is highly unlikely that any cellular DNA that might have contaminated the isolated plasma membranes possessed a significant amount of sequence homology to viral

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**Chart 6.** An aliquot of Fractions 11 to 15 of the gradient shown in Chart 5 was analyzed by isopyknic centrifugation in alkaline Cs$_2$SO$_4$ (pH 12). Trichloroacetic acid-precipitable radioactivity was measured after addition of 100 µg yeast RNA and an equal volume of 10% trichloroacetic acid.

**Chart 7.** A pooled aliquot of the Fractions 11 to 15 of Chart 5 (hybrid region) was denatured in alkali to eliminate the unlabeled viral RNA. This was then neutralized and hybridized back again with a different sample of DNA.

**Chart 8.** Another aliquot of the material used for the experiment shown in Charts 5 to 7 was analyzed by alkaline sucrose gradient centrifugation (4 to 20% sucrose, pH 12). The scale of apparent molecular weights was obtained as described for Chart 4.

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60 to 70 S $^{32}$P-labeled RSV-RNA. The result indicates that most of the $^3$H-labeled DNA synthesized in the plasma membrane in vivo and banded in the hybrid region in the 1st fractionation is rehybridizable with viral RNA suggesting that the DNA products are the initial copy of viral RNA.
RNA, since no such DNA was found in plasma membranes from uninfected cells. The DNA synthesized in vivo and hybridizable to viral RNA is heterogeneous in size whereas dTMP-32P DNA produced in vitro is homogeneous and of low molecular weight as described earlier in the present study. This difference can be best explained by assuming that the joining of the newly synthesized fragments occurs in vivo but the necessary polynucleotide ligase activity (11) is lost during isolation of plasma membrane.

Weisbach et al. (15) reported that infection with leukovirus RAV-2 leads to the formation of large amounts of viral RNA-dependent DNA polymerase activity whose properties differ from the cellular RNA-dependent DNA polymerase normally present in CE. The latter enzyme cannot copy viral RNA. In our experiments the activity for DNA synthesis complementary to viral RNA and associated with plasma membranes was generated only by a new infection with an active virus on a susceptible strain of cells. From these findings we assume that the enzyme responsible for viral transcription is not a cellular DNA polymerase normally present in the cell but a viral enzyme introduced or induced de novo by new infection.

The present experiments cannot eliminate the possibility of nuclear involvement (4) in the initial transcription of viral RNA. Chromosomal DNA synthesis is so dominant in our unsynchronized cell systems that synthesis of viral DNA in the nucleus, if it occurs, would not be observed in our autoradiographic studies.

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REFERENCES


Figs. 1 to 3. Electron microscopic autoradiographs of 3T3/BALB cells infected with MSV for 1 hr and pulse labeled with TdR-3H (125 @Ci/ml) for an additional 1 hr (Fig. 1) and 2 hr (Figs. 2 and 3). Photopositive grains are found on the peripheral portion of cytoplasm or the cell surface which was characteristically divided into finger-like fine processes and attached to neighboring cells. The cytoplasm contains some phagocytic vesicles (ph). Fig. 1, x 12,000; Fig. 2, x 35,000; Fig. 3, x 20,300.

Fig. 4. In 3T3/BALB cells labeled with TdR-3H between 6 and 10 hr after MSV infection, the photopositive grains are seen to be distributed evenly in this electron microscopic autoradiograph. The characteristic labeling of the plasma membrane area is no longer observed at this stage of infection. x 17,700.

Fig. 5. The plasma membrane (A), mitochondria (B), and microsomes (C) with ribosomal particles free (r) or associated with membrane were isolated from 3T3/BALB cells infected with MSV for 1 hr. Each of the fractions were incubated to test for their ability to support DNA synthesis in vitro. In the fractions no intact virions or Mycoplasma were seen.
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