Isolation and Characterization of a Virus-Specific Ribonucleoprotein Complex from Reticuloendotheliosis Virus-Transformed Chicken Bone Marrow Cells

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Chicken bone marrow cells transformed by reticuloendotheliosis virus (REV) produce in the cytoplasm a ribonucleoprotein (RNP) complex which has a sedimentation value of approximately 80 to 100S and a density of 1.23 g/cm³. This RNP complex is not derived from the mature virion. An endogenous RNA-directed DNA polymerase activity is associated with the RNP complex. The enzyme activity was completely neutralized by anti-REV DNA polymerase antibody but not by anti-avian myeloblastosis virus DNA polymerase antibody. The DNA product from the endogenous RNA-directed DNA polymerase reaction of the RNP complex hybridized to REV RNA but not to avian leukemia virus RNA. The RNA extracted from the RNP hybridized only to REV-specific complementary DNA synthesized from an endogenous DNA polymerase reaction of purified REV. The size of the RNA in the RNP is 30 to 35S, which represents the subunit size of the genomic RNA. No 60S mature genomic RNA was found within the RNP complex. The significance of finding the endogenous DNA polymerase activity in the viral RNP in infected cells and the maturation process of 60S virion RNA of REV are discussed.

Reticuloendotheliosis viruses (REVs) are a group of avian retroviruses which are distinct from the avian leukemia-sarcoma viruses (12, 17, 20, 22, 29). This group of viruses consists of four members: reticuloendotheliosis virus strain T (REV-T), duck spleen necrosis virus, duck infectious anemia virus, and chick syncytial virus (25). These are C-type particles which possess a single-stranded RNA genome (17, 20, 25) and an endogenous RNA-directed DNA polymerase activity (15, 24, 31).

The four members of REVs are very closely related in terms of nucleotide sequence of the RNA (17, 18), antigenicity of their DNA polymerase (22, 23), group-specific antigens (21), and their morphology (19).

Although much information has accumulated regarding the mature virions of REV, very little is known about the intracellular stage of the virus precursor before budding. We used REV-T-transformed chicken bone marrow cells (BMC) to study the intracellular viral precursor. This cell line has been cloned and shown by biochemical assay and electron microscopic study to produce REV-T continuously under normal culture conditions (9, 15, 19). The haploid genome of BMC contains approximately five copies of REV genome (manuscript in preparation). The REV-T produced from the BMC contains no detectable avian leukemia virus (ALV) by hybridization studies (15), and the endogenous RNA-directed DNA polymerase activity in the REV-T from BMC is not neutralized by antiserum against the DNA polymerase of Rous sarcoma virus–Rous-associated virus-0 [RSV- (RAV-0)] (31). These properties, together with the ability to grow in suspension culture to high density, make the BMC suitable for the study of intracellular viral precursor of REV.

A search for intracytoplasmic viral precursor structures by thin-section electron microscopy using BMC revealed no viral substructure in the cells. Using biochemical methods, we have isolated from the cytoplasm of the BMC a virus-specific ribonucleoprotein (RNP) complex which has properties markedly different from, and which appears to be the precursor of, the mature virion. This report describes the characterization of this complex with regard to its physical properties, the size and specificity of its genetic content, and its associated DNA polymerase activity.

MATERIALS AND METHODS

Cells and viruses. REV-T-transformed chicken BMC were a kind gift from H. Bose, University of
Texas at Austin. The BMC were derived from an infected bird and have been cloned and characterized (9). These cells were grown in suspension in Dulbecco-modified Eagle medium, pH 6.9 (GIBCO), supplemented with 7.5% fetal calf serum. The BMC produce REV-T under normal culture conditions. REV-T was harvested every 12 h, concentrated by ultracentrifugation, and purified by equilibrium density gradient centrifugation in 10 to 65% (wt/vol) sucrose gradients. B77 strain of avian sarcoma virus and Rous-associated virus-61 (RAV-61) were obtained from H. M. Temin. Primary chicken embryo fibroblasts (CEF) from 12-day-old chicken embryos (SPAFAS, Norwich, Conn.) were prepared by standard techniques previously described (1). The CEF cells were grown in Temin-modified Eagle medium (International Scientific Industries) supplemented with 20% tryptose phosphate broth and 5% fetal calf serum.

Endogenous RNA-directed DNA polymerase assay. The endogenous RNA-directed DNA polymerase reaction was carried out in a reaction mixture containing 80 μM dATP, dCTP, and dGTP, 60 μM ATP, 80 μg of pyruvate kinase/ml, 20 μg of phosphoenolpyruvate/ml, 2.5 mM MnCl₂, 40 mM KCl, and about 2.75 nM [3H]dTTP (58 Ci/mmol) (Schwarz/Mann) in 20 mM Tris-hydrochloride buffer (pH 8.0) containing 5 mM dithiothreitol and 100 μg of actinomycin D/ml. Samples from fractions of sucrose gradient analysis of virus-specific components in BMC were assayed in a final volume of 100 μl at 37°C for 60 min, unless otherwise stated in the figure legends. After incubation, a portion of each mixture was spotted onto filter paper presaturated with 0.1 M sodium pyrophosphate. Trichloroacetic acid-p precipitable radioactivity was then determined.

Preparation of 3H-labeled complementary DNA (1H)cDNA products from the endogenous reaction. The endogenous DNA polymerase activity was provided by either Nonidet P-40 (NP-40)–dissociated virions or the extracted RNP complex from BMC with the use of the same conditions as in the DNA polymerase assay. Incubation was carried out at 37°C for 1 h in a final volume of 5 ml. At the end of the incubation, the labeled DNA products were purified as previously described (17, 18).

Isolation of RNP complex from REV-transformed chicken bone marrow cells. Amounts of 1 × 10⁹ to 2 × 10⁹ actively growing REV-T-transformed BMC were harvested and washed twice by centrifugation in ice-cold phosphate-buffered saline (PBS) at 300 × g for 10 min. The cell pellet was resuspended in 1 ml of reticulocyte standard buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.01 M NaCl, 0.0015 M MgCl₂). Cells were homogenized by applying 15 strokes in a tight-fitting glass Dounce homogenizer which was heated in an oven to destroy ribonuclease activity. Nuclei and cellular membranes were separated from the microsomal fraction by two consecutive centrifugations at 5,500 × g for 10 min each at 5°C. The cytoplasmic fraction was then treated with EDTA to a final concentration three times that of Mg²⁺ ions in reticulocyte standard buffer to dissociate polysomes before being overlaid onto 10 to 30% (wt/vol) sucrose gradients in Beckman SW41 centrifuge tubes. Separation of cytoplasmic components was accomplished by centrifugation at 40,000 rpm for 120 min with a Beckman SW41 rotor. Intracellular RNP complex was detected by assaying each collected fraction for endogenous RNA-directed DNA polymerase activity. The sedimentation coefficient of the enzyme-associated material was determined by comparing it with the polyribosomoprobe of mouse L929 cells in a parallel gradient, which was monitored by UV absorption. Fractions containing the highest endogenous RNA-directed DNA polymerase activity were pooled for the synthesis of DNA products or for preparation of RNA for use in hybridization experiments. To separate the RNP complex by density, cytoplasmic extracts from BMC were layered onto 30 to 70% sucrose gradients. Centrifugation was carried out at 40,000 rpm for 34 h at 5°C. Intracellular RNP was detected by endogenous RNA-directed DNA polymerase assay as described above.

To insure that the enzyme-associated RNP complex found in the cytoplasm of BMC was not due to degradation of mature virions in the intracytoplasmic vacuoles and/or on the membranes during the homogenization procedure, equal amounts of purified REV-T (approximately 200 μg of proteins) were added to about 2 × 10⁸ BMC or the same number of uninfected normal CEF just before homogenization. The cytoplasmic extracts were prepared from both of these preparations in exactly the same manner as the control BMC. The cytoplasmic fractions were then analyzed with 10 to 30% (wt/vol) sucrose gradients formed on top of 70% (wt/vol) sucrose cushions in Beckman SW50-1 centrifuge tubes. Centrifugation was carried out at 49,000 rpm for 100 min at 5°C with a SW50-1 rotor. Amounts of 20 μl from each gradient fraction were assayed for endogenous RNA-directed DNA polymerase activity in the presence of 0.1% NP-40. The nuclei and membrane pellets were resuspended in 0.15 ml of PBS containing 0.2% NP-40. Samples of 60 μl from each of these nuclei and membrane fractions were assayed for endogenous RNA-directed DNA polymerase activity to detect any membrane-associated virus.

Antibody neutralization. The anti-REV DNA polymerase antiserum and the anti-avian myeloblastosis virus (AMV) DNA polymerase antiserum were kind gifts from S. Mizutani and H. M. Temin, University of Wisconsin (22, 23). To test for the specificity of DNA polymerase in the RNP complex from the cytoplasm of BMC, the isolated RNP complex was divided into 100-μl portions and treated separately with anti-REV DNA polymerase antiserum (50 μg of immunoglobulin G), anti-AMV DNA polymerase antiserum (50 μg of immunoglobulin G), or with a mixture of both (50 μg of each). Rabbit-anti-rat antiserum made against normal rat kidney cells was used as a control. Incubation was carried out at room temperature for 45 min. After incubation, the RNA-directed DNA polymerase activity was determined by using the endogenous reaction as described above.

Nucleic acid hybridization. [3H]cDNA products were synthesized from either purified REV-T or from the intracellular RNP complex isolated from BMC, by use of the endogenous RNA-directed DNA polymerase reaction. The phenol-chloroform procedure for extraction of DNA and RNA from cells or purified
virus followed the previously published method (17). Liquid-phase hybridization techniques were used for all hybridization experiments (17). The extent of hybridization was determined by S1 nuclease digestion (17, 28).

**Nascent DNA products analysis.** Nascent [3H]DNA products were prepared from the intracellular RNP complex from the BMC by use of the endogenous RNA-directed DNA polymerase reaction as described above. After incubation for 10 min at 37°C, the reaction was terminated by adding sodium dodecyl sulfate and EDTA to the final concentrations of 0.5% and 50 mM, respectively. Phenol-extracted yeast RNA (500 µg) was added as carrier, and the products were extracted twice with phenol and three times with chloroform. [3H]-labeled DNA products were precipitated in ethanol overnight at −20°C. After pelleting by centrifugation at 8,000 × g for 10 min, [3H]-labeled DNA products were redissolved in TSE buffer (0.1 M NaCl, 0.001 M EDTA, in 0.02 M Tris-hydrochloride, pH 7.3) and either directly analyzed by centrifugation in 10 to 30% (wt/vol) sucrose gradients or treated with RNase A or sodium hydroxide before analysis in sucrose gradients. Conditions for sucrose gradient sedimentation analysis are described in the figure legends.

To show that the size and the integrity of the RNA template in the intracellular RNP were not altered during the incubation and extraction procedure, a small amount (approximately 50 µg of proteins) of NP-40-disrupted REV-T was mixed with the extracted RNP. The 10-min [3H]DNA products from the mixture were analyzed in sucrose gradients, and sedimentation values were compared to 32P-labeled mature REV-T viral RNA.

**RESULTS**

**Isolation of a REV-specific RNP complex from REV-T-transformed chicken bone marrow cells.** Preliminary experiments showed that the cytoplasmic extracts of REV-T-transformed BMC had an endogenous RNA-directed DNA polymerase activity similar to that of mature virions. Since REV has been found to mature by budding not only through the cytoplasmic membranes but also into the intracytoplasmic vacuoles (19), the DNA polymerase-associated cytoplasmic components which may represent virion precursors must be separated from the intravacuolar virosomes by use of rate-zonal or equilibrium density gradients. To isolate the cytoplasmic viral components, the cytoplasmic extracts were prepared from actively growing REV-T-transformed BMC and treated with EDTA to dissociate polyribosomes. The cytoplasmic components were then separated by centrifugation in a 10 to 30% (wt/vol) sucrose gradient. Viral components in the gradient were detected by assaying each collected fraction for endogenous RNA-directed DNA polymerase activity (Fig. 1).

The majority of the endogenous DNA polymerase activity was found in the 80 to 100S region. A lower level of activity sedimented near the top of the gradient. Uninfected normal CEF did not contain the enzyme activity which sedimented in the 80 to 100S region, although there was some enzyme activity near the top of the gradient. The latter observation is in accordance with previous observations of uninfected chicken embryo cells (16). The enzyme activity in the 80 to 100S region is not from the intravacuolar virosomes because the mature virion has a sedimentation coefficient of about 600S and therefore would be pelleted at the bottom of the gradient.

To rule out the possibility that the enzyme-associated 80 to 100S material was produced from the mature intravacuolar REV-T during the homogenization and extraction procedures, purified REV-T was added to the BMC or to uninfected normal CEF before homogenization. These were then processed identically as the control BMC, which were not mixed with virus, to obtain the cytoplasmic fractions. The cytoplasmic fractions were analyzed by centrifugation in 10 to 30% (wt/vol) sucrose gradients formed on top of 70% (wt/vol) sucrose cushions. Viral components in the gradients were moni-
stored by assaying a sample from each fraction by use of the endogenous RNA-directed DNA polymerase assay in the presence of 0.1% NP-40 (Fig. 2).

BMC alone contained the RNA-directed DNA polymerase activity which sedimented in the region of 80 to 100S. There was a very small amount of mature virion at the interface above the 70% sucrose cushion (Fig. 2a). In the cytoplasmic fraction from the BMC premixed with purified REV-T, large amounts of mature virions were detected at the interface on the 70% sucrose cushion while the polymerase activity of the 80 to 100S material was unchanged (Fig. 2b). In contrast, when REV-T was added to normal CEF and the extracted cytoplasmic fraction was analyzed, no detectable enzyme-associated material was sedimented at 80 to 100S. Furthermore, we were surprised to find very few mature virions on the 70% sucrose cushion (Fig. 2c). This initially puzzling phenomenon was resolved when the nuclei and membrane pellets were assayed for RNA-directed DNA polymerase activity (Fig. 2d). Most of the purified REV-T added to the normal CEF was recovered in the membrane fraction (represented by bar c’ in Fig. 2d), whereas a large amount of the REV-T added to the BMC was recovered from the interface of the 70% sucrose (Fig. 2b) but very few of the virions became associated with the cellular

Fig. 2. Sedimentation pattern of RNA-directed DNA polymerase activity in the cytoplasm of REV-T-transformed chicken bone marrow cells (BMC) and normal chicken embryo fibroblasts (CEF) mixed with purified REV-T. Cytoplasmic extracts were prepared from $2 \times 10^8$ BMC or CEF as described in Materials and Methods after purified REV-T (approximately 200 µg of proteins) was mixed with the cells just before homogenization. The cytoplasmic fractions were treated with EDTA and centrifuged at 49,000 rpm for 100 min at 5°C in 10 to 30% (wt/vol) sucrose gradients made on top of 70% (wt/vol) sucrose cushions. A sample of 20 µl from each collected fraction was assayed for endogenous RNA-directed DNA polymerase activity in the presence of 0.1% NP-40. (a) control BMC without addition of REV-T. (b) BMC mixed with REV-T. (c) CEF mixed with REV-T. In panel d, the nuclei and membrane pellets from BMC (a’), BMC + REV-T (b’), and CEF + REV-T (c’) were resuspended in phosphate-buffered saline with 0.2% NP-40. A 60-µl sample from each of these fractions was assayed for endogenous RNA-directed DNA polymerase activity. Enzyme activity was expressed as counts per minute of $[^{3}H]$dTMP incorporated during 60 min of incubation at 37°C.
membrane (bar b). This suggests that the cell surface of the BMC is already saturated with viral proteins, whereas the normal CEF provides free virus-binding sites for the added virions.

These experiments conclusively showed that the 80 to 100S enzyme-associated material from the cytoplasm of the BMC was not derived from mature REV-T during the homogenization procedure, but represented a distinct entity.

The density of the DNA polymerase-associated intracytoplasmic material was determined by equilibrium density gradient centrifugation in a 30 to 70% (wt/vol) sucrose gradient (Fig. 3). The endogenous RNA-directed DNA polymerase activity in the total cytoplasmic extract was resolved into two peaks, one having a density of 1.23 g/cm³ and the other having a density of 1.16 g/cm³ (Fig. 3a). The 1.23 g/cm³ band corresponds to the density of nucleocapsid of mature virion. The 1.16 g/cm³ material may well represent mature virions trapped between the cells or in the intracytoplasmic vacuoles. Most of this low-density material could be removed by washing the cells three or four times before homogenization. When the pooled fractions of the 80 to 100S material from the velocity gradient as shown in Fig. 1 were analyzed in the equilibrium density gradient, only one homogeneous peak corresponding to 1.23 g/cm³ was observed (Fig. 3b). This indicated that the enzyme-associated 80 to 100S material was identical to the material having a density of 1.23 g/cm³ and appeared to be an RNP complex present within the cytoplasm of the transformed cells.

REV-specific endogenous RNA-directed DNA polymerase activity in the RNP. Since the genome of normal CEF contains endogenous sequences of avian leukosis virus (ALV) (29), it is necessary to demonstrate that the RNA-directed DNA polymerase activity detected in the RNP complex in the BMC is specific for REV and not due to the expression of the endogenous ALV sequences. The 80 to 100S RNP complex was isolated from REV-T-transformed BMC by use of rate-zonal centrifugation in sucrose gradient. The RNP complex was divided into four portions which were treated separately with anti-REV DNA polymerase antiserum, anti-AMV DNA polymerase antiserum, or a mixture of anti-REV and anti-AMV DNA polymerase antisera. Rabbit anti-rat antiserum (made against normal rat kidney cells) was used as a control. The treated fractions were then assayed for endogenous RNA-directed DNA polymerase activity. Kinetics show that anti-REV DNA polymerase antiserum can specifically neutralize the RNA-directed DNA polymerase activity associated with the RNP complex (Fig. 4). Neither the anti-AMV DNA polymerase antiserum nor the anti-rat antiserum affects the enzyme activity. Results from the reciprocal experiments showed that the anti-AMV polymerase antiserum completely neutralized the polymerase activity of B77 avian sarcoma virus (data not shown). Since the anti-AMV DNA polymerase antiserum completely neutralized the polymerase activity of the endogenous RAV-0 (22) and B77 avian sarcoma virus DNA polymerase activity but not the DNA polymerase activity of REV, the endogenous DNA polymerase activity found in the RNP is not due to the expression of ALV.

REV-specific RNA in the RNP isolated from the BMC. To demonstrate the presence of REV-specific RNA in the intracellular RNP, we extracted RNA from pooled 80 to 100S RNP complex and hybridized the RNA with [3H]-cDNA synthesized from purified REV-T, using the liquid phase hybridization technique de-

![Fig. 3. RNA-directed DNA polymerase activity in cytoplasmic fraction of REV-T-transformed chicken bone marrow cells resolved by equilibrium density sucrose gradient centrifugation. Cytoplasmic extract from 2 x 10⁸ chicken bone marrow cells was treated with EDTA and centrifuged at 40,000 rpm for 34 h in a 30 to 70% (wt/vol) sucrose gradient at 5°C. Each collected fraction was assayed for endogenous RNA-directed DNA polymerase activity (a). Alternatively, the RNP complex was pooled from the 80 to 100S region from a velocity sucrose gradient and centrifuged at 40,000 rpm for 34 h in a 30 to 70% (wt/vol) sucrose gradient. Each fraction was assayed for endogenous RNA-directed DNA polymerase activity (b). Enzyme activity was expressed as counts per minute of [3H]dTMP incorporated during 60 min of incubation at 37°C (●), density in grams per cubic centimeter (○).]
VOL. 28, polymerase was RNP inized at a rected Fig. 5, rRNA's represents RNA. REV-specific sucrose different with were expressed with ated and tature material is RNA in the commented from made from was incorporated RNA hybridization of cDNA probe cDNA nonspecific out because experiment showed that the vast majority of RNA in the 80 to 100S region of the gradient represents rRNA's because monosomes cosedimented at this region (data not shown). To rule out nonspecific hybridization, the same REV-T cDNA probe was hybridized with RAV-61 RNA. No hybridization was observed. Furthermore, no hybridization was observed when the RNA extracted from RNP was incubated with cDNA made from RAV-61. This shows that the hybridization between the RNP RNA and REV-T cDNA was specific.

To further substantiate that the 80 to 100S RNP complex is REV specific, we prepared [H]cDNA from the endogenous DNA polymerase reaction of the 80 to 100S RNP. This RNP [H]cDNA was hybridized to the REV-T viral RNA. Maximal hybridization is approximately 40% at a C value of 2 \times 10^{-1} \text{ mol}\cdot\text{ml}^{-1}\cdot\text{s}^{-1} (Fig. 6). Since the virion RNA used in the hybridization was extracted from virus produced by REV-T-transformed BMC, it was necessary to demonstrate that the REV-T was free from the endogenous ALV contamination. RAV-61 [H]-cDNA was hybridized with the RNA from the REV-T and showed no hybridization, indicating that there was no detectable amount of ALV in the virus preparation. The results demonstrate that the RNP complex contains RNA which can be transcribed by the RNP-associated DNA polymerase into cDNA. This cDNA specifically hybridizes to purified REV-T RNA.

It is noteworthy that the hybridization using

![Figure 4](http://jvi.asm.org/) Antiserum neutralization of the endogenous RNA-directed DNA polymerase activity associated with the intracellular RNP from REV-T-transformed chicken bone marrow cells. Viral RNP was isolated from cytoplasmic fraction of REV-T-transformed chicken bone marrow cells by velocity sucrose gradient centrifugation similar to that in Fig. 1. Equal portions of the isolated RNP were treated with different antibodies for 45 min at room temperature and then assayed for endogenous RNA-directed DNA polymerase activity. Enzyme activities were expressed as counts per minute of [H]dTMP incorporated at various times. Anti-rat antisera (O); anti-AMV DNA polymerase antisera (Q); anti-REV DNA polymerase antisera (\Delta); mixture of both anti-REV DNA polymerase and anti-AMV DNA polymerase antisera (\Delta).

![Figure 5](http://jvi.asm.org/) Kinetics of hybridization of [H]cDNA synthesized from REV-T to RNA extracted from the intracellular RNP. [H]cDNA was prepared from a 1-h product of endogenous DNA polymerase reaction of purified REV-T in the presence of 100 \mu g of actinomycin D/ml. Amounts of 2,000 cpm per sample of the REV-T [H]cDNA were annealed with 6 \mu g of REV-T viral RNA/ml (O), 6 \mu g of RAV-61 viral RNA/ml (\square), and 36 \mu g of RNA/ml from the RNP isolated from cytoplasm of REV-T-transformed chicken bone marrow cells (\Delta). In addition, 2,000 cpm per sample of RAV-61 [H]cDNA was hybridized to 120 \mu g of RNA/ml from the RNP isolated from REV-T-transformed chicken bone marrow cells (\Delta). The hybridization was performed at 63°C in 25 \mu l of DNA-RNA annealing buffer (0.5 M NaCl, 0.1% sodium dodecyl sulfate, 0.001 M EDTA, 2% phenol, and 0.05 M Tris-hydrochloride, pH 7.3) sealed in 25-\mu l micropipettes. Samples were withdrawn at different times and the extent of hybridization was determined by S, nuclease digestion. The results were expressed as the proportion of total [H]cDNA hybridized at a given value of C greater than 0.5 M salt. Approximately 8.5% of the REV-T [H]cDNA and 4% of the RAV-61 [H]cDNA were resistant to S, nuclease in a control sample incubated without RNA. These have been subtracted from the plotted values.
40 WONG AND KANG

Fig. 6. Kinetics of hybridization of [3H]cDNA synthesized from intracellular RNP from REV-T-transformed chicken bone marrow cells to REV-T viral RNA. [3H]cDNA was prepared from a 1-h product of endogenous DNA polymerase reaction of pooled intracellular RNP in the presence of 100 μg of actinomycin D/ml. Amounts of 2,000 cpm per sample of the RNP [3H]cDNA were annealed with 6 μg of REV-T viral RNA/ml (C), or 6 μg of yeast RNA/ml (D). In addition, 2,000 cpm per sample of [3H]cDNA synthesized from RAV-61 was hybridized with 6 μg of REV-T viral RNA/ml (△). Conditions of hybridization and determination of the extent of hybridization were the same as in Fig. 5. Approximately 7.8% of the RNP [3H]cDNA was resistant to S1 nuclease in the control sample incubated by itself and has been subtracted from plotted values. Approximately 90% of 32P-labeled RAV-61 RNA became resistant to ribonuclease digestion after annealing to about 30-fold excess of RAV-61 DNA products.

[3H]cDNA prepared from the RNP would only proceed to 40% of the maximum when hybridized with homologous viral RNA. This apparent low efficiency in hybridization is not due to the presence of DNA of opposite polarities in the [3H]cDNA preparations, because hybridization still proceeded to only about 40% when the same [3H]cDNA probes made from the RNP were hybridized to cellular DNA of REV-transformed BMC (data not shown). Similar results were observed when [3H]cDNA synthesized from REV-T was hybridized to REV-T RNA (15). The significance of this is still unclear.

Size of virus-specific RNA in the RNP complex. To determine the size of the RNA template in the RNP complex, RNA was extracted from the 80 to 100S RNP and separated in a 10 to 30% (w/vol) sucrose gradient by rate-zonal centrifugation. A sample from each collected fraction of the gradient was hybridized to a constant amount of [3H]cDNA made from purified REV-T. Figure 7 shows a prominent peak of virus-specific RNA that sedimented at approximately 30 to 35S. In addition, small proportions of other RNA species were observed to sediment both faster and slower than the major RNA species. This result suggests that the size of the major species of RNA in the RNP complex is 30 to 35S.

Analysis of the nascent DNA products from the endogenous DNA polymerase reaction of the RNP. To determine the size of the RNA template in the RNP complex, we also analyzed the RNA-template-associated nascent DNA products. [3H]DNA products were synthesized for 10 min from the 80 to 100S RNP by use of the endogenous DNA polymerase reaction. After phenol-chloroform extraction, the nascent [3H]cDNA products recovered from the aqueous phase were analyzed in a 10 to 30% (w/vol) sucrose gradient by rate-zonal centrifugation. Under non-denaturing conditions, a distinct population of the nascent DNA products sedimented in the 30 to 35S region, and the vast majority of DNA sedimented near the top of the gradient (Fig. 8a). The 30 to 35S peak could be removed when the initial products were treated with RNase A or 0.5 N NaOH (Fig. 8b and c). This indicates that the DNA sedimenting in the

Fig. 7. Sedimentation pattern of REV-specific RNA in the intracellular RNP from REV-T-transformed chicken bone marrow cells. Intracellular RNP was isolated from cytoplasmic fraction of REV-T-transformed chicken bone marrow cells by use of sucrose gradient sedimentation velocity centrifugation similar to that shown in Fig. 1. RNA was extracted from the pooled RNP material and subjected to centrifugation at 49,000 rpm for 90 min at 5°C in a 10 to 30% (w/vol) sucrose gradient in TSE buffer. An equal sample from each collected fraction was hybridized with 1,000 cpm of [3H]cDNA synthesized from purified REV-T in a 50-μl final volume of DNA-RNA hybridization buffer. Hybridization was performed at 63°C for 100 h. Extent of hybridization was determined by digestion with S1 nuclease. Approximately 3% of the [3H]cDNA was resistant to S1 nuclease in a control sample incubated without RNA and was subtracted from the plotted values. [3H]cDNA was used as markers (arrow).
RNP COMPLEX FROM REV-TRANSFORMED CELLS

Fig. 8. Sedimentation analysis of nascent DNA products from the endogenous DNA polymerase reaction carried out with intracellular RNP from REV-T-transformed chicken bone marrow cells. Intracellular RNP was isolated from REV-T-transformed chicken bone marrow cells by use of sucrose gradient sedimentation velocity centrifugation similar to that shown in Fig. 1. The pooled RNP was used for endogenous RNA-directed DNA polymerase reaction. \[^3H\]cDNA synthesis was allowed to proceed for 10 min at 37°C in the presence of 100 µg of actinomycin D/ml. The 10-min DNA products were either analyzed directly with 10 to 30% (wt/vol) sucrose gradients in TSE buffer centrifuged at 49,000 rpm for 90 min (a), treated with 50 µg of RNase A per ml at 37°C for 30 min before sedimentation analysis (b), or treated with 0.5 N NaOH at 63°C for 60 min before sedimentation analysis (c). \[^3H\]cDNA was used as markers (arrows).

To demonstrate that the 30 to 35S RNA was

subunit size of the REV RNA is about 35S (12), it is therefore conceivable that the RNP complex contains only the subunit size of the viral genome, which may represent the intracellular precursor of the genomic RNA.

We conclude that the RNA template in the RNP complex has a sedimentation value of about 30 to 35S, which is consistent with the result from our hybridization study shown in Fig. 7. Since the

Fig. 9. Sedimentation analysis of nascent DNA products from intracellular RNP mixed with disrupted REV-T. Nascent \[^3H\]cDNA products were synthesized as described in Fig. 8, except that the RNP was mixed with purified REV-T disrupted with 0.2% NP-40. The 10-min DNA products were either analyzed directly with 10 to 30% (wt/vol) sucrose gradients in TSE buffer centrifuged at 49,000 rpm for 90 min (a) or treated with 50 µg of RNase A per ml at 37°C for 30 min before sedimentation analysis (b). \[^3P\]labeled purified REV-T viral RNA was analyzed in a parallel gradient (c). \[^3H\]cDNA was centrifuged in another parallel gradient to serve as markers (arrows).
actually the immature form and not derived from a degradative product of the mature viral genome. NP-40-disrupted purified REV-T was added to the intracellular RNP, and 10-min DNA products were synthesized from the mixture by use of the endogenous RNA-directed DNA polymerase reaction. The [3H]DNA products were extracted and analyzed by sucrose gradients similar to those shown in Fig. 9. The nascent DNA synthesized in this reaction sedimented at three distinct places (Fig. 9). The hybrids that sedimented between 50 and 60S were undoubtedly produced from the mature REV-T genome that was added because it had a sedimentation value similar to that of 35P-labeled REV-T viral RNA (Fig. 9c). This peak was not observed in DNA synthesis when the intracellular RNP alone was used. Therefore, we conclude that the 30 to 35S RNA template in the RNP complex is not derived from degradation of the mature virion RNA. Identical results were obtained with the 1.23 g/cm2 RNP complex isolated from equilibrium density sucrose gradients instead of from velocity sedimentation gradients (data not shown).

DISCUSSION

We have isolated an RNP complex from the cytoplasm of REV-T-transformed chicken BMC which contains endogenous RNA-directed DNA polymerase activity. This RNP complex is found only in the REV-transformed cells and not in the normal cells. The RNP complex has a density of 1.23 g/cm2 and sediments in the 80 to 100S region in sucrose gradients. These properties indicate that it is distinct from the mature virion. The sedimentation pattern of the RNP is not altered by mixing the BMC with virus during homogenization. Also, the RNP peak cannot be created by homogenizing normal CEF mixed with purified REV-T. These results strongly indicate that the RNP is not a degradative product resulting from the homogenization and extraction procedures. Previous studies show that the core isolated from REV has a density of 1.25 to 1.26 g/cm2 (4), slightly heavier than our density determination of the cytoplasmic viral RNP complex. The RNP complex possesses properties that suggest that it is in a diffused form. It has a relatively slow sedimentation rate and slightly lighter density compared to the mature viral core. Also, the absence of any discernible viral substructure in the cells when studied by electron microscopy seems to support the same conclusion (19).

Endogenous RNA-directed DNA polymerase assay shows that the intracytoplasmic RNP complex is fully functional and able to synthesize REV-specific complementary DNA in vitro. However, it is not yet clear whether the RNP complex is constantly synthesizing proviral DNA in the transformed cells, where stably integrated proviral DNA is already present in the cellular genome. If this is indeed the case, the newly synthesized viral DNA from the RNP could account for the presence of linear viral DNA duplexes characteristic of acute infection up to several weeks after infection in the cytoplasm of avian sarcoma virus-infected duck cells (31), avian sarcoma virus-infected quail tumor cells (11), and mouse mammary tumor virus-infected rat cells (26).

On the other hand, our findings are in contrast to that of David Baltimore, Massachusetts Institute of Technology, who found that the RNA-directed DNA polymerase activity of murine leukemia virus was not fully functional until the virus was released from the cell (personal communication).

Since all chicken cells contain endogenous ALV sequence in their DNA genome, it is important to demonstrate that the RNP complex in the cytoplasm of the REV-transformed chicken BMC is specific for REV. Antibody neutralization studies showed that the endogenous RNA-directed DNA polymerase activity associated with the RNP complex could be neutralized only by anti-REV DNA polymerase antisemur but not by anti-AMV DNA polymerase antisemur, indicating that the enzyme in the RNP complex was specific for REV. This is in accordance with the previous finding that REV-T purified from the BMC contained only REV-specific polymerase (32).

When cDNA synthesized from the endogenous reaction of RNP was hybridized with viral RNA extracted from purified REV-T, up to 40% of the DNA probe was hybridized. The kinetics of this hybridization were similar to that when the probe was made from purified REV-T (15). In both cases the maximal levels of hybridization were about 40%. In contrast to the endogenous polymerase reaction, the cDNA probe made from an exogenous reaction using purified REV RNA and purified ALV DNA polymerase could anneal completely to REV RNA (17). The incomplete hybridization is not due to some cDNA which is in the same polarity as the virion RNA since annealing with DNA from infected cells does not increase the extent of hybridization (data not shown). We also know that the non-annealing portion of the cDNA does not hybridize with an excess amount of virion RNA.

In three separate sets of hybridization experiments between the cDNA made from the REV-T and REV-T virion RNA at RNA concentrations of 6, 12, and 20 µg/ml, the maximal levels
of hybridization were the same as that shown in Fig. 6.

It is possible that the cDNA contains a significant proportion of short DNA transcripts. Since the melting points of hybrids between virion RNA and the short cDNA should be low, the extent of hybridization may not reach 100% under stringent hybridization conditions. Another possibility is that the probe contains some homopolymers synthesized by a terminal transferase-like activity. This possibility is, however, highly unlikely because the endogenous RNA-directed DNA polymerase reaction requires all four nucleoside triphosphates and very little DNA is synthesized in the absence of any one nucleoside triphosphate (15).

To study the size of intracytoplasmic virus-specific RNA, we used two methods. In the first approach, REV-specific RNA was detected by hybridization after the RNA isolated from the RNP was subjected to velocity centrifugation. This method gave an estimated sedimentation coefficient of about 30 to 35S for the major species of REV-specific RNA in the RNP complex.

Study of the size of the RNA in the RNP complex had been complicated by the fact the RNP cosedimented with 80S ribosomes. Labeling experiments with [3H]uridine and [32P], showed that the RNA in the 80 to 100S material contained a majority of 28S and 18S rRNA (data not shown). Thus, the virus specific RNA could be detected only by the hybridization method. We found some other sizes of RNAs which hybridized with REV cDNA in addition to the 30 to 35S RNA. Of these, we often observed two minor peaks at about 27S and 21S. These may represent contamination by subsets of mRNA's comparable to those found in avian sarcoma virus- and murine leukemia virus-infected cells (2, 7, 8, 10, 14, 27, 30). More difficult to explain are the fast-sedimenting RNAs between 40 and 50S. The significance of these is still not clear, although virus-specific RNA of larger than subunit size has been found in cells infected by Rous sarcoma virus (14) and murine leukemia virus (13).

In the second approach, the sedimentation value of RNA-cDNA complex, which represented the RNA template-nascent DNA product, was determined. The nascent DNA products were found to be in short fragments of less than 10S in sedimentation value. Therefore, the sedimentation value of the RNA-cDNA complex would be largely determined by the RNA moiety. The RNA-cDNA complex was found to have a sedimentation value of 30 to 35S based on 19 separate determinations. Since the subunit size of REV genome is about 35S (12), it is thus concluded that the size of the RNA in the intracellular RNP complex is equivalent to one subunit of the RNA genome of the virus. Studies on the maturation of Rous sarcoma virus and murine leukemia virus have shown that viral RNA found in the cytoplasm of the infected cells and in newly budded virions consists mainly of 30 to 35S RNA subunits, whereas mature virions contain the 60 to 70S genomic RNA (5–8, 14, 30). Our findings with REV-T-transformed cells seemed to be in accordance with these findings.

Our results suggest that the REV-T-transformed chicken BMC produce an RNP complex which contains an endogenous RNA-directed DNA polymerase activity. This RNP complex contains REV-specific DNA polymerase and REV-specific RNA template, and may represent the virion precursor. The size of the RNA in the RNP complex is equivalent to the subunit size of the virion genomic RNA. No 60S mature genomic RNA was found in the intracellular RNP complex. Thus, the formation of mature viral genomic RNA seems to occur after the budding, probably during the condensation of the nucleoid. More recent data on the protein composition of the RNP complex indicate that it contains several virus-specific proteins, including some not found in the mature virion, which may represent the precursor and intermediate proteins (unpublished data). Moreover, virus assembly appears to begin when the 30 to 35S RNA is in the cytoplasm. Internal proteins such as the polymerase can complex with the viral RNA at this stage to form a functional RNP complex which also contains the primer molecule since it is capable of endogenous transcription.

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LITERATURE CITED


