Vol. 116, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1983 Pages 303-311

DEMONSTRATION OF FREE REVERSE TRANSCRIPTASE IN THE NUCLEI OF EMBRYONIC TISSUES OF THE JAPANESE QUAIL

H. Mondal and P. H. Hofschneider

Max-Planck-Institute of Biochemistry, Dept. Virusforschung, 8033 Martinsried, near Munich, F.R.G.

Received September 2, 1983

SUMMARY: Reverse transcriptase is detected in the nuclei as well as the cytoplasm of normal uninfected Japanese quail embryonic tissues. At least 60 % of the nuclear enzyme occurs in the free-form, whereas 85 % of the cytoplasmic enzyme is bound to particles having a buoyant density equivalent to retroviral cores (1.19 - 1.22 g/ml). The enzyme is purified by ion-exchange chromatography and glycerol sedimentation gradients. It prefers oligo(dG).poly(C) in Mg^{2+} to all other synthetic template-primers, and transcribes poly(A) containing RNAs. Information present in the above particles is endogenous indicating that RT is not derived from exogenous retrovirus.

Alu repetitive DNA sequences and pseudogenes, specially the pseudogenes of small nuclear RNAs (snRNAs) of the U-series reported in the genomes of higher animals and human beings, are suggested to be generated through RNA intermediates by the process of reverse transcription (1, 2 and for review see ref. 4). This hypothesis suggests that reverse transcriptase (RT) exists at least temporarily in the cells of higher animals and human beings as a physiological requisite, and not only as a consequence of induction of proviral genes or of infection with exogenous retroviruses. Furthermore, for this hypothesis to be valid, RT should be present not only in the cytoplasmic retrovirus-like particles as reported by this laboratory (5 - 8) and elsewhere (9 - 12) but also in the nuclei of germ-line cells, preferably in the free-form or in the core (lipoprotein coat-less retrovirus)-bound form to facilitate reverse transcription (2). We demonstrate here that in very early embryos of Japanese quails RT is present in the nucleus and cytoplasm in the free- as well as in the core-bound form. This is the first demonstration of RT in the nuclei of normal cells supporting the above hypothesis.

MATERIALS AND METHODS

Materials

AMV and AMV-RT was obtained from Life Science Inc. (Fl. USA) and [^{3}H] dGTP (sp. act. 20 Ci/mmole); [^{3}H]dTTP (sp. act. 18.8 Ci/mmole) from New England Nuclear Corp. (Boston, Mass.) Fertilised Japanese quail eggs were obtained from Brüterei Ludwig Hölzl, Moosburg (FRG). The embryos used here as a source of material were ALV-negative, chf-negative and ALV-group-specific antigen-negative.

0006-291X/83 $1.50

Cell-fractionation-procedures

The embryos were collected aseptically and placed in ice-cold LS-buffer. All the operations thereafter were performed at 0 - 4 °C and freezing of samples at any stages was strictly omitted. The embryos were washed twice with LS-buffer, and homogenized (1 - 2 strokes) in the presence of the above buffer with a Dounce homogenizer fitted with a loose pestle. The resultant embryonal tissues were washed twice with TNM buffer and once again homogenized (20 - 25 strokes) in the presence of 5 volumes of TNM buffer, with a Dounce homogenizer fitted with a tight pestle. The nuclei were pelleted at 800 x g for 10 min, washed twice with TNM buffer containing 0.02 % NP-40 (Nonidet - P 40) and finally rewashed twice with TNM buffer only. The resultant nuclei were suspended in 5 volumes of 0.7 M sucrose in TNM buffer layered on 2.3 M sucrose in TNM buffer and finally centrifuged down at 40,000 rpm for 1 hr in a SW 41 rotor. The pelleted nuclei were incubated in TNM buffer for 10 min at 0 - 4 °C and finally washed once with the above buffer. The nuclear preparation thus obtained was free from cytoplasmic membrane as monitored with a phase-contrast microscope. The nuclei (unless otherwise mentioned) were homogenized with a Branson Sonifier for 1 - 2 min at 20 - 25 Watts in TNM buffer. The sonicated material was centrifuged at 14,000 rpm for 1 hr to pellet the nucleoli. The nucleoplasm was centrifuged at 100,000 x g for 1 hr. The resultant pellets are referred to here as nuclear particles and the supernatant as particle-free-nucleoplasm. The cytoplasm obtained after cell homogenization was centrifuged at 14,000 rpm for 10 min. The pellet was discarded and the supernatant was further centrifuged at 100,000 x g for 1 hr. The resultant pellet is referred to here as cytoplasmic particles and the supernatant as particle-free cytoplasm.

Isolation and Purification of RT

RTs were purified separately both from the particle-free nucleoplasm and cytoplasmic particles. However, cytoplasmic or nuclear particles were purified further through equilibrium density sucrose gradients as described earlier (8), except that the gradients were run in TNM buffer. Gradient purified particles and nucleoplasm were extracted separately with 0.1 % NP-40 and 0.5 M KCl at 0 - 4 °C for 2 hr, and desalted through a G-75 column (2 x 10 cm) preequilibrated with buffer A. Enzyme activities eluted at the void volume were pooled and applied on a DEAE cellulose column. Fractions were monitored for RT activities and peak fractions were pooled and layered on a phosphocellulose column after adjusting the pH to 7.0 and the KCl concentration to 100 mM. The PC column purified samples were concentrated to 1 ml with 30 % polyethyleneglycol in buffer C and dialysed overnight against buffer A, containing 5 % glycerol, before being applied to a 5 - 20 % linear sucrose gradient in buffer C. The peak fractions with RT were pooled and dialysed overnight against buffer A.

Assay Methods

Assays with different synthetic template-primers and with activated DNA were performed as described earlier (9). Assays with RNA and endogenous template were performed maintaining the ratios Mg : total dNTPs concentration (0.9 : 1.0) as described previously (8). NP-40 was omitted from the reaction mixtures throughout the experiments (unless otherwise stated). The acid-precipitable cpm in each enzyme assay was corrected for background cpm, obtained by an identical reaction conducted with heat denatured enzyme from the corresponding fraction, which ranged between 500 to 1.000.

Buffers

TNM buffer: Tris-HCl, pH 7.4, 10 mM; NaCl, 100 mM; $MgCl_2$, 10 mM. TNE buffer: Tris-HCl, pH 8.0, 10 mM; NaCl, 100 mM; EDTA, 1 mM. TNE buffer: Tris-HCl, pH 8.0, 10 mM; NaCl, 100 mM; EDTA, 10 mM. LS-buffer: Tris-HCl, pH 7.4, 10 mM; NaCl, 10 mM; DTT, 1 mM; $MgCl_2$, 10 mM. Buffer A: Tris-HCl, pH 8.0, 10 mM; KCl, 10 mM; PMSF, 0,01 mM; DTT, 1 mM; glycerol, 20 %. Buffer B: Tris-HCl, pH 7.0, 10 mM; KCl, 100 mM; PMSF, 0,01 mM; DTT, 1 mM; glycerol 20 %. Buffer C: Tris-HCl, pH 8.0, 50 mM; KCl, 500 mM; PMSF, 0,01 mM; DTT, 1 mM; glycerol, 25 %.

RESULTS AND DISCUSSION

Kinetics of expression of RT

The kinetics of expression of RT in various embryonic tissues from Japanese quails were investigated in order to ascertain whether a correlation exists between RT-expression and embryonic differentiation as described previously for human tissues (8). The results are shown in Table 1. RT is first detected in 2-day-old embryos. It increases up to the 6th day and then decreases sharply until no activity is detected in fully developed embryos at 13 days. The peaks of RT activity in isolated organs indicate a correlation with the differentiation of organs (i. e. in relatively early differentiating heart and liver tissues). This suggests that RT is involved in some physiological process of differentiating cells.

Subcellular localization and status of the RT

To test the subcellular distribution of RT, we fractionated the cells from tissues of 4-day-old embryos as described in the method sections and the fractions were assayed for RT. RT can be detected in the nucleoplasm as well as in the cytoplasm (Table 2). At least 25 - 30 % of total intracellular RT activity is found in the nuclei, and 60 % of the nuclear activity is not pelletable at 100,000 x g. This clearly indicates that part of the RT in embryonic cells is present in the nuclei and a major portion of

Table 1. Kinetics of expression of RT in quail embryonal tissues at different ages.

Embryo age (in days)	Particle-bound RT activity (pmoles of dGMP incorporation in response to oligo(dG).poly(C)) in tissues of 10 embryos.						
	Brain	Heart	Liver	Kidney	residual tissues	total Activity	Activity/g of Embryonal tissues
1	-	-	-	-	-	Nil	NT
2	-	-	-	-	-	20	50
3	-	-	-	-	-	100	1,200
4	520	230	-	-	1,400	2.150	1,430
6	760	380	1,600	1,420	245	4,405	1,110
8	448	120	48	48	20	684	9
10	50	110	16	172	Nil	348	NT
13	Nil	Nil	Nil	5	Nil	5	NT
16	Nil	Nil	Nil	Nil	Nil	Nil	NT

Organs are not dissectable in 1- and 3-day-old embryos. Residual tissue refers to the remainder of the embryo after removal of the above mentioned organs for which the RT was measured. The RT activity was measured as described in the text, using cytoplasmic retroviral-like particles purified through equilibrium density sucrose gradients. Assuming that enzyme was synthesized in the cytoplasm and as the majortiy of the cytoplasmic RT was bound to the particles, only cytoplasmic particles were used here to measure the expressed RT in the cells. The data presented above are the total RT in respective organs from 10 embryos, and comprise the average of three such sets of experiments using 10 embryos. Protein was estimated as described earlier (13).

Table 2. Distribution and Status of the Enzyme.

Localization	RT activity (pmoles of dGMP incorporation in response to oligo(dG).poly(C)) in tissues of 10 embryos		
	Free-form	core-bound	
		assayed in the presence of NP-40	assayed in the absence of NP-40
Nucleus (washed with NP-40)	558	372	360
Nucleus (not washed with NP-40)	550	412	407
Cytoplasm	291	1980	2010

Whole 4-day-old embryos were fractionated as described in the methods. 4-day-old embryos were used here, because RT activity / g of tissue was highest at this stage (see Table 1), however, distribution patterns of RT in 5- and 6-day-old embryos were more or less the same as observed in day 4. The RT activity was measured as described in methods, from cytoplasm and particle-free nucleoplasm without any further purification, however, cytoplasmic and nuclear particles were purified through equilibrium sucrose gradients as described in the methods because, without purification, hardly any RT activity is detectable in the particle fractions. NP-40 whenever used in the assay mixtures was 0.1 %. The data presented above are the total RT in respective fractions from 10 embryos, and comprise the average of three such set of experiments using 10 embryos each.

this exists in free-form. Concerning the presence of RT in the nuclei it may be argued that RT is bound to cytoplasmic membrane attached to the nuclei. However, a NP-40 wash of the nuclear fraction as shown in the Table 2 does not effect the RT activity in this fractions. Therefore, it can be excluded that this activity is due to a contamination of the nuclear fraction with cytoplasmic membrane. As to the status of the enzyme, specially in the case of the nuclear preparation, it may be argued that NP-40 treatment to the nuclei releases the enzyme from the particles. However, nuclear preparations with or without NP-40 treatment show almost the same amount of free-form RT (Table 2, line 2). Moreover, it may be mentioned here that a cytoplasmic preparation which has never been treated with NP-40, contains free-form enzyme. It is also been shown in Table 2 that 40 % of the nuclear and 85 % of the cytoplasmic enzyme are pelleted at 100,000 x g. However, for measuring RT activities in the pellet fractions, NP-40 treatment is unnecessary. This indicates that retroviruses (if there are any) in the pellet fractions are without lipoprotein coat.

The RT containing particles in the pellet fractions have the characteristic density of C-type retroviral cores (Fig. 1). However, based on

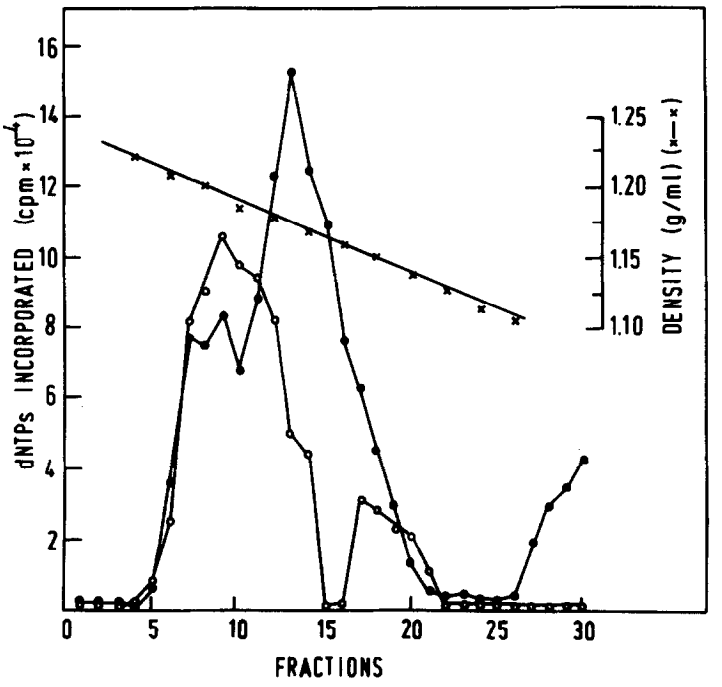

Figure 1. Distribution of retroviral-like particles through equilibrium density sucrose gradients. 100,000 x g pellet fraction of cytoplasm obtained from 15 g embryonal tissue were suspended in 1 ml TNM buffer, layered on six density sucrose gradients (10 - 60 % w/w) in TNM buffer, centrifuged and ultimately fractionated after centrifugation (200,000 x g for 18 hr in a SW 41 rotor) as previously described (8). Fractions were monitored for enzyme activities with oligo(dG).poly(C), (O—O) and with activated DNA, (●—●) as described in the method sections. In both cases, Mg^{2+} was used as divalent cation and [^{3}H]dGTP (sp. activity 20 Ci/mmole) as substrate, however, in case of activated DNA, the 3 other unlabeled nucleotides (5 μM each) were used. The data presented above show the activities in 10 μl aliquots from each fractions (0.38 ml). 100,000 x g pellet of nuclear fractions centrifuged under identical conditions showed a similar density pattern (data not shown).

the density alone, it should not be inferred that RT reported here is associated with retroviral cores. DNA polymerase α attached to the membrane also bands at this density (data not shown). Furthermore, the nucleic acids present in the particles do not hybridize to the RNAs of avian retroviruses as shown earlier (7) nor do the particles show infectivity (data not shown). For these reasons, we prefer to term them as retroviral elements, not as retroviral particles.

Purification and characterization of RT

The RT activities from the nucleoplasm as well as from the cytoplasmic particles were purified through DEAE cellulose, phosphocellulose column chromatography and glycerol sedimentation gradients (data not shown) as described in the methods. In both cases, RT elutes at 0.28 M KCl from DEAE cellulose and at 0.25 M KCl from phosphocellulose columns. Both free-

Table 3. Template-primer specificities of RT.

Exp. No.	Template	Primer	Conditions	Enz.act.(nmole/mg of protein) Particle-bound	Free-form
1	poly(C)	oligo(dG)	Mg/40 mM KCl	94.50	19.00
2	poly(C)	oligo(dG)	Mn/40 "	42.32	8.10
3	poly(A)	oligo(dT)	Mg/100 "	23.62	3.00
4	poly(A)	oligo(dT)	Mn/100 "	28.35	2.00
5	poly(dA)	oligo(dT)	Mg/20 "	1.89	0.90
6	poly(dA)	oligo(dT)	Mn/20 "	0.94	1.00
7	-	oligo(dG)	Mn/100 "	0	0
8	DNA-activated	-	Mg/40 "	67.75	22.00
9	DNA-activated	+ Act. D	Mg/40 "	0.94	0.50
10	AMV-RNA	-	Mg/140 "	1.00	0.20
11	AMV-RNA	oligo(dT)	Mg/140 "	20.79	3.10
12	AMV-RNA	oligo(dT) + Act. D	Mg/140 "	14.17	1.50
13	SnRNA	-	Mg/140 "	3.20	0.38

Activity in 10 µl aliquots of sedimentation glycerol gradient purified samples was measured in each cases as described in the text. [^{3}H]dTTP was used as a substrate in case of template-primers: oligo(dT).poly(A); oligo (dT).poly(dA). [^{3}H]dGTP was used in the cases of other template-primers including activated DNA and RNA, however, in the reaction mixtures containing RNA and DNA, 3 other unlabeled nucleotides were added. Therefore, for calculating the nucleotide incorporation in case of RNA and DNA templated reactions, the actual value was multiplied by a factor of 4 assuming the equimolar incorporation of all four substrates, and presented above.

form and particle-bound enzymes have mol. wt. 110,000. The gradient purified RT was then characterized by its preference for different synthetic and natural template-primers (Table 3). Like AMV-RT, the RT from quail embryonic tissue preferred oligo(dG).poly(C) over all other synthetic template-primers. It also preferred Mg^{2+} to Mn^{2+}. Oligo(dT).poly(dA) was accepted with much lower efficiency and oligo(dG) was not accepted at all. Heteropolymeric RNA was accepted by this RT as well as by AMV-RT (data not shown). This pattern of utilization of different template-primers by these enzymes either isolated from nucleus or from cytoplasm excludes the possibility that it represents one of the other eukaryotic DNA polymerases (i. e. α, β and γ), deoxyribonucleotidyl terminal transferase or bacterial DNA polymerase and prove that these are RT.

Immunological properties of the quail-RT

RTs isolated both from the nucleus and cytoplasm have been tested for immunological relatedness to different retroviral RTs and DNA polymerase γ. Both forms of quail-RTs are marginally related (20 - 30 %) to AMV-RT but not at all to other retroviral RTs and DNA polymerase γ (Fig. 2). Thus from Table 3 and Fig. 2 it may be deduced that both forms of RTs in the nucleus and in the cytoplasm are biochemically similar, and they occur in free- as well as core-bound forms.

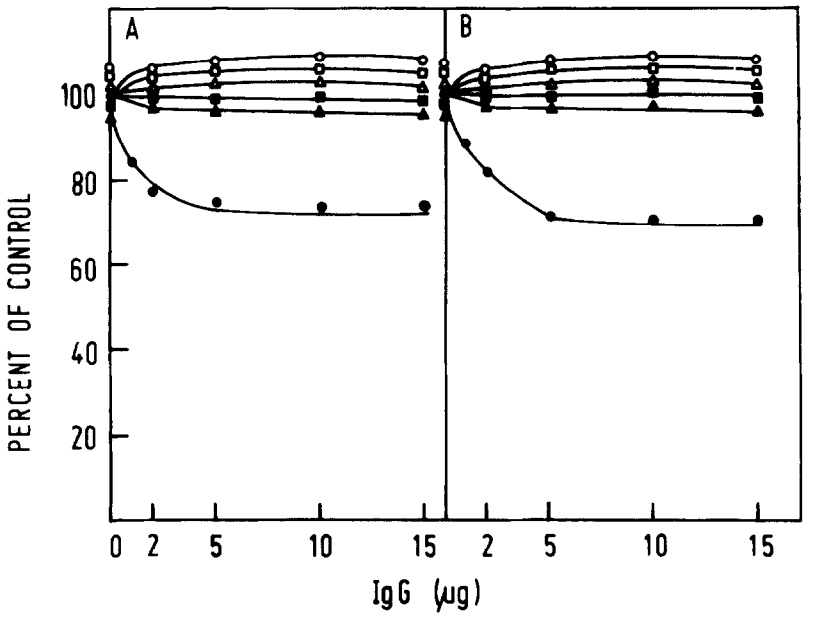

Figure 2. Neutralization test of Quail-RT by antiseras of RT of different Retroviruses and DNA pol γ . Increasing amount of IgG were preincubated for 4 hr at 0 - 4 °C with fixed amount of glycerol gradient purified RTs. Assay for incorporation of [^{3}H]dGMp on oligo(dG).poly(C) template with preincubated samples was performed as described in the methods. From 80,000 - 100,000 cpm in case of particle-bound enzyme and 12,000 - 20,000 cpm in the case of nuclear free-form enzyme were observed in samples preincubated in the absence of IgG. The values are expressed as percentage of activity of a duplicate sample that was preincubated similarly in the absence of IgG. RT from the following viruses: AMV (Avian myeloblastosis virus), (●-●); SNV (Spleen necrosis virus), (○-○); RD114 (Endogenous virus of cat cells), (△-△); GALV (Gibbon ape leukemia virus), (▲-▲); and DNA pol γ, (■-■); Non-Immune IgG, (□-□). The neutralizing capacities of antiseras for homologous enzymes were tested and found strongly positive (data not shown). Panel A: Cytoplasmic particle-bound enzyme; Panel B: Nuclear free-form enzyme. The antiseras were kindly provided by Dr. R.C. Gallo (National Cancer Institute, Bethesda, MD, USA) and Prof. H. Temin (McArdle Laboratory, Madison Wisconsin, USA).

Origin of the nucleic acids of the particles

RT is bound to a particle which like retroviral cores bands at 1.20 g/ml (Fig. 1). This suggests that the RT reported above may have derived from retroviruses. To explore this possibility, cDNAs (without the sequences annealing to rRNA and tRNA which were removed by hydroxylapatite chromatography) to the particle's RNAs were hybridized to adult quail chromosomal DNA. The kinetics of the hybridization presented in Fig. 3, show a sigmoidally shaped Cot curve. Part of the particle-cDNA anneals to cellular DNA below 10 Cot. This indicates that part (30 % of the input cpm) of cDNAs are homologous to highly repetitive sequences other than rRNA and tRNA genes present in the quail genome. Fig. 3 also shows that another part of the cDNAs (50 % of the input cpm) anneals (80 %) to the cellular genome at

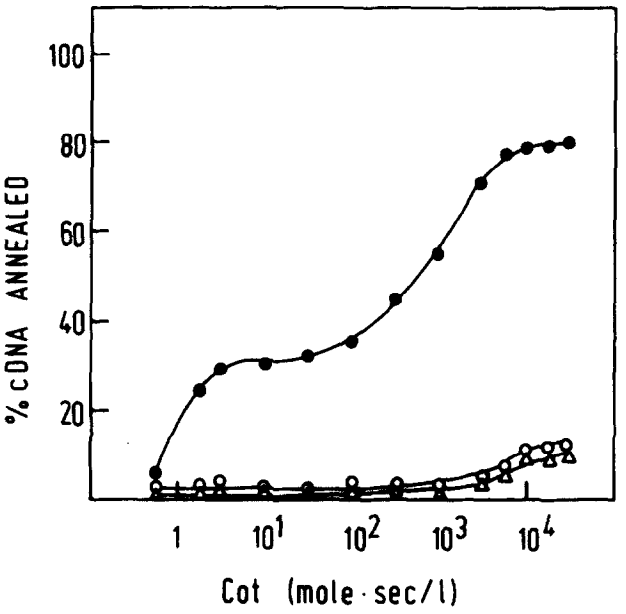

Figure 3. Annealing of different cDNAs with adult quail cell DNA. RNAs were extracted by phenol : chloroform : isoamylalcohol from purified retroviral particles as previously described (7). It was further purified through hydroxylapatite chromatography in 50 % formamide at 20 °C. cDNA probes corresponding to RNA of retroviral particles (0.14 M potassium phosphate buffer, pH 7, eluant) and to the RNA of AMV and RAV-0 were prepared by cellulose impregnation technique as previously described (8). Annealing was carried out as previously described (8) using cellular DNA at 10 mg/ml and [^{3}H]-cDNA to quail RNA, AMV- and RAV-0-RNA each at 0.5 µg/ml (3,000 cpm per reaction) in a 10 µl volume in a sealed capillary tube as previously described (7). Quail cell DNA were annealed to: cDNA of quail particle, (●–●); cDNA of AMV, (○–○); cDNA of RAV-0 (Δ–Δ).

10^4 Cot. This suggests that the particle is associated with some RNAs which are transcribed from single copy sequences. In identical experiments with AMV-cDNA and with RAV-0-cDNA, it is shown in Fig. 3 that: 1) in the case of particle-cDNA, hybridization kinetics below 10 Cot is not due to sequences common to avian viruses; 2) the quail stock used here is not infected with AMV or RAV-0 virus (endogenous virus). The results presented above clearly indicate that the information carried by the particles containing RT is endogenous, but not related to RAV-0.

Similar particle-bound- and free-form-RT can be isolated from the nuclei of 5-day-old duck embryos (unpublished observation). Based on this result and the data from the quail embryos, it may be speculated that, as long as the RT is not bound to a particle it is available for reverse transcription of cellular self-primed RNAs and the binding of RT to the above mentioned particles (though coatless) is a measure to restrict random reverse transcription. In addition, the particles themselves may act as moveable genetic elements like intracisternal A particles in mouse system (14) but at pre-

sent, we do not have any evidence supporting the latter hypothesis. Now it would be of great interest to show that some self-primed RNAs (3) are reverse transcribed *in vivo*. Studies along these lines are in progress.

ACKNOWLEDGEMENT

We thank Miss V. Baumgartner for her technical assistance.

REFERENCES

1. Van Arsdell, S.W., Denison, R.A., Bernstein, L.B., Weiner, A.M., Manser, T., Gesteland, R.F. (1981) Cell 26, 11-17.
2. Jagadeeswaran, P., Forget, B.G., and Weissman, S.M. (1981) Cell 26, 141-142.
3. Bernstein, L.B., Mount, S.M., and Weiner, A.M. (1983) Cell 32, 461-472.
4. Sharp, P. (1983) Nature 301, 471-472.
5. Bauer, G., and Hofschneider, P.H. (1967) Proc. Natl. Acad. Sci. U.S.A. 73. 3025-3029.
6. Bauer, G., Friis, R.R., Jilek, G., and Hofschneider, P.H. (1978) Biochim. Biophys. Acta 518, 125-137.
7. Mondal, H., Jendis, J., and Hofschneider, P.H. (1980) in Viruses in naturally occuring cancer (Essex, M., Todaro, G., and zur Hausen, H., eds.), Vol. 7, pp. 1239-1250, Cold Spring Harbour Laboratory, Cold Spring Harbor, New York.
8. Mondal, H., and Hofschneider, P.H. (1982) Int. J. Cancer 30, 281-287.
9. Mondal, H. (1977) Biochim. Biophys. Res. Commun. 79, 67-75.
10. Bauer, G., and Temin, H.M. (1979) J. Virol. 29, 1006-1013.
11. Lueders, K., Leder, A., Leder, P., and Kuff, E. (1982) Nature 295, 426-428.
12. Nelson, J., Leong, J., and Levy, J.A. (1982) Proc. Natl. Acad. Sci. U.S.A. 75, 6263-6267.
13. McKnight, G.S. (1977) Anal. Biochem. 78, 86-92.
14. Kuff, E.L., Feenstra, A., Lueders, K., Smith, L., Hawley, R., Hozumi, N., and Shulman, M. (1983) Proc. Natl. Acad. Sci. U.S.A. 80. 1992-1996.