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An RNA-dependent DNA polymerase, different from the known viral reverse transcriptases, in the chicken system

(leukosis-virus-free eggs/allantoic fluid/template-primer complexes/DNA·RNA hybridization/IgG neutralization test)

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ABSTRACT **The properties of an RNA-dependent DNA polymerase (an RNA-dependent DNA nucleotidyltransferase), which occurs ubiquitously in the allantoic fluid of uninfected, leukosis-virus-free eggs, are described. It is shown that the enzyme can synthesize faithful transcripts from natural RNA (globin mRNA). By biochemical and immunological methods, the enzyme can be clearly distinguished from the reverse transcriptases of the known chicken RNA tumor viruses and therefore seems to be a member of a so far unknown class of chicken polymerases.**

Since the finding of reverse transcriptase (1, 2), the function of this enzyme for the replication of RNA tumor viruses and for the transformation of cells by these viruses has been clearly demonstrated (3). A role for reverse transcription in the normal physiology of cells has been postulated (4). However, no enzyme has been isolated from virus-free cells that, after purification, exhibits the biochemical functions of RNA-dependent DNA polymerase [RNA-dependent DNA nucleotidyltransferase (reverse transcriptase)]. In the chicken system three different DNA polymerases have been isolated from uninfected cells: DNA polymerase α and β (5) and polymerase γ (6, 7). These polymerases can use poly(A)·$(dT)_{12}$ as a template, but cannot use other synthetic ribonucleotide strands or natural RNA as templates. Further, enzyme purified from a fraction with endogenous RNA-dependent DNA polymerase activity (8) did not have the characteristics of reverse transcriptase *in vitro*, but resembled polymerase α or β.

Therefore, in the chicken system appearance of an enzyme with the ability to use templates such as poly(C)·$(dG)_{12}$ or natural RNA was always connected with a virus of one of the two groups of known chicken RNA tumor viruses, the avian leukosis/sarcoma viruses (ALV/ASV) or the reticuloendotheliosis viruses (REV).

Investigations of the polymerases in the allantoic fluid of embryonated leukosis-virus-free eggs showed the ubiquitous presence of RNA-dependent DNA polymerase. The enzyme is bound to particles and therefore preliminarily termed "particle enzyme". The properties of these particles will be described elsewhere*. Here we report some of the characteristics of this enzyme and its differentiation from the known polymerases in the chicken system.

MATERIALS AND METHODS

Materials. Avian myeloblastosis virus (AMV) was generously supplied by Dr. J. Beard, St. Petersburg. Purified REV, strain T, was kindly provided by Dr. K. Moelling and Dr. R. Friis, University of Giessen. IgG purified from the serum of rats that had been immunized with purified reverse transcriptase from AMV was a generous gift of the laboratories of Dr. R. Gallo, Bethesda, and Dr. S. Spiegelman, New York. Leukosis-virus-free SPF-VALO eggs were a gift of Dr. E. Vielitz, Lohmann Tierzucht, Cuxhaven. Tests were also performed with eggs obtained from SPAFAS Inc., Connecticut, and with eggs from a special flock of gs^- chf^- chickens, originating from SPAFAS Inc., and helpfully provided by Dr. P. Duesberg, Berkeley.

Enzyme Purification. Particles were purified from the allantoic fluid of 10-day-old embryonated chicken eggs, as described in the legend to Fig. 1. This particle fraction was investigated for the presence of leukosis viruses of subgroup A–G by Dr. R. Friis. He could not detect helper or interfering activity, both of which are typical properties of ALV/ASV. On sodium dodecyl sulfate-polyacrylamide gels the purified particle fraction gives a pattern completely different from ALV/ASV or REV*. In radioimmune assays, kindly performed by Dr. A. Vaheri, University of Helsinki, the major structural component of ALV/ASV, p 27, could not be detected. Reverse transcriptases were purified and the enzymes were separated by sedimentation gradient centrifugation as described by Markus *et al.* (9).

The IgG Neutralization Assay was as described by Watson *et al.* (10), except that the preincubation of the enzyme with IgG was at 0–4° for 12 hr.

Standard Polymerase Assay. The final concentrations were 10 mM magnesium acetate, 20–80 mM KCl, 50 mM Tris·HCl (pH 8.3), 10 mM dithioerythritol, and 1 mM ATP. The usual assays of 100 μl contained 5 μCi of labeled deoxynucleoside triphosphate (8 Ci/mmol), 1 μg of template-primer complex, and 50 μg of bovine serum albumin (A grade). When heteropolymeric nucleic acid was used as template, the unlabeled deoxynucleoside triphosphates were each at a concentration of 0.6 mM.

The reaction mixture was incubated at 37° and trichloroacetic acid precipitable radioactivity was measured.

RESULTS

Characteristics of the Enzyme. The purified enzyme was tested with a set of template-primer complexes, which allow the differentiation of reverse transcriptase from other DNA polymerases (see Fig. 1). The enzyme can use the primer-template complexes poly(C)·$(dG)_{12}$, poly(A)·$(dT)_{12}$, and poly(dC)·$(dG)_{12}$ quite well, whereas poly(dA)·$(dT)_{12}$ is used much less efficiently. Primer alone gives no detectable activity. This pattern of utilization of synthetic homopolymers by the enzyme excludes terminal deoxynucleotidyltransferase and polymerases α, β, and γ, and suggests that the enzyme is a reverse transcriptase. Comparative studies with AMV polymerase and the same set of template-primer complexes gave almost identical results (data not shown).

To demonstrate that the enzyme can use natural, heteropo-

Abbreviations: ALV/ASV, avian leukemia/sarcoma viruses; AMV, avian myeloblastosis virus; REV, reticuloendotheliosis virus.

* G. Bauer and P. H. Hofschneider, in preparation.

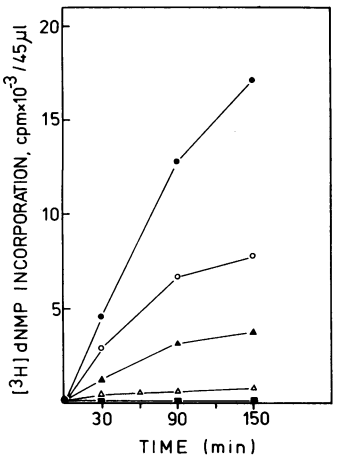

FIG. 1. The allantoic fluid of 10-day-old embryonated eggs was clarified from cells and cell debris by low-speed centrifugation. It was then centrifuged in a fixed-angle rotor (rotor 30) at 27,000 rpm for 45 min at 4°. The pellets were resuspended in STE buffer [0.1 M NaCl, 0.01 M Tris·HCl (pH 8.0), 0.001 M EDTA] and layered onto a discontinuous sucrose gradient in a Beckman SW 27 rotor. The material was centrifuged through 20% sucrose onto a 50% cushion (26,000 rpm, 2.5 hr). The material above the 50% sucrose was collected, and after dilution layered onto a continuous sucrose gradient, ranging from 20 to 35% sucrose (in STE buffer). It was centrifuged for 50 min at 26,000 rpm. The gradient was collected from the bottom and the fractions were tested in standard polymerase assays in the presence of 0.1% Nonidet P-40 and poly(C)·$(dG)_{12}$ as template. The active fractions (upper part of the gradient) were diluted and layered onto a continuous sucrose gradient, ranging from 20 to 50% sucrose. It was centrifuged in rotor SW 41 at 30,000 rpm for 16 hr. The material was collected from the bottom and tested for RNA-dependent DNA polymerase activity. The active fractions were diluted in STE buffer and pelleted in a Ti 50 fixed-angle rotor. At this stage the enrichment factor was about 10,000 and the material was free of poly(dA)-dependent activity, which is present in excess in the first pellet. Per milliliter of allantoic fluid, an enzyme activity was obtained that incorporated 10,000–20,000 cpm of [^{3}H]dGMP (8 Ci/mmol) per hr in a poly(C)-dependent reaction. The pelleted material was processed for isolation of the enzyme as described by Markus *et al.* (9). The enzyme, after the affinity chromatography step, was tested in standard polymerase assays with the indicated template-primer complexes and the respective labeled complementary deoxynucleoside triphosphate. (●) Poly(C)·$(dG)_{12}$; (○) poly(dC)·$(dG)_{12}$; (▲) poly(A)·$(dT)_{12}$; (△) poly(dA)·$(dT)_{12}$; (■) $(dG)_{12}$.

lymeric RNA templates for reverse transcription, we studied the utilization of globin mRNA by the enzyme. Globin mRNA, with $(dT)_{12}$ hybridized to the poly(A) strand of the 3′ end of the molecule, is a good template for reverse transcriptases (11). To be sure of measuring the reverse transcription of the heteropolymeric region of the RNA molecule, we used [^{3}H]dGTP as labeled monomer. As shown in Table 1, the particle enzyme uses globin mRNA in comparison to poly(C)·$(dG)_{12}$ as efficiently as the AMV enzyme. The full activity is dependent on the presence of template, primer, and all four deoxynucleoside triphosphates, and can be completely inhibited by RNase A. Actinomycin D has no clear effect on the reaction, indicating that it does not proceed further than hybrid synthesis under these conditions. The remaining activity in the absence of primer is probably due to priming oligonucleotides contained in the globin mRNA fraction. In any case, a terminal transferase activity can be excluded, since the enzyme does not use primers without templates (Fig. 1).

To prove that a faithful transcript had been synthesized, we analyzed the purified reaction product on Cs_2SO_4 density gradients. The radioactivity was found in an intermediate position between RNA and DNA density, demonstrating that the product is a DNA·RNA hybrid (Fig. 2A). After alkaline treatment the radioactivity shifted to the region of DNA density, indicating that DNA had been synthesized (Fig. 2B). This DNA sediments at approximately 5 S (Fig. 3). After hybridization of the alkali-treated material to an excess of newly added globin mRNA (5 µg/100 µl of reaction mixture), the radioactivity is found between the RNA and hybrid positions of the density gradient (Fig. 2C). This series of experiments shows that the enzyme synthesized a DNA transcript of the heteropolymeric region of the globin mRNA, and therefore may be termed "reverse transcriptase". Additional data show that the primary product of the globin mRNA-directed reaction remains at hybrid density after S_1 nuclease treatment and has a melting temperature of 82°, as compared to 66° for poly(A)·poly(dT). The product formed after backhybridization has a melting temperature of 85°. After S_1 nuclease treatment, the product shifts from a density between that of RNA and hybrid to that of complete hybrid, as expected for an RNA·DNA hybrid composed of an RNA molecule and a relatively shorter DNA molecule*.

Table 1. Response of reverse transcriptase from particles or AMV to natural, heteropolymeric RNA

Template	Primer	Reaction conditions	cpm [^{3}H]dGMP incorporated by: Particle enzyme	AMV enzyme
Globin mRNA	$(dT)_{12}$	Complete	3,300	3,800
Globin mRNA	$(dT)_{12}$	Complete + actinomycin D	2,870	
Globin mRNA	$(dT)_{12}$	Complete + RNase	0	
Globin mRNA	—	Complete	866	
Globin mRNA	$(dT)_{12}$	−dCTP	880	
Globin mRNA	$(dT)_{12}$	−dATP	0	
	$(dT)_{12}$	Complete	0	
Poly(C)	$(dG)_{12}$	Complete	101,000	96,200

Aliquots of purified enzymes were incubated in the presence of 2.5 µg of rabbit globin mRNA and 1 µg of $(dT)_{12}$ per 100-µl reaction mixture. Conditions were as described for the standard polymerase assay in *Materials and Methods*, with the addition of unlabeled dATP, dCTP, and dTTP to a final concentration of 0.6 mM each. The only labeled deoxynucleoside triophosphate was [^{3}H]dGTP (8 Ci/mmol), at a final concentration of 0.012 mM. Actinomycin D and ribonuclease A were at a final concentration of 50 µg/ml. Incubation at 37° was for 160 min for the particle enzyme and 40 min for the AMV enzyme. The acid-insoluble radioactivity was determined from the total volume. For comparison, identical assays were run in the presence of poly(C)·$(dG)_{12}$ instead of globin mRNA.

Distinction of the Enzyme from Reverse Transcriptases of Known Chicken RNA Tumor Viruses. Since, in the chicken system, reverse transcriptase has only been found in tumor viruses or in cells infected by these viruses, we had to exclude the possibility that the activity isolated originated from a contaminating or endogenous RNA tumor virus. We therefore compared the enzyme activity with the reverse transcriptases of the two known chicken RNA tumor viruses, i.e., the REV and ALV/ASV groups.

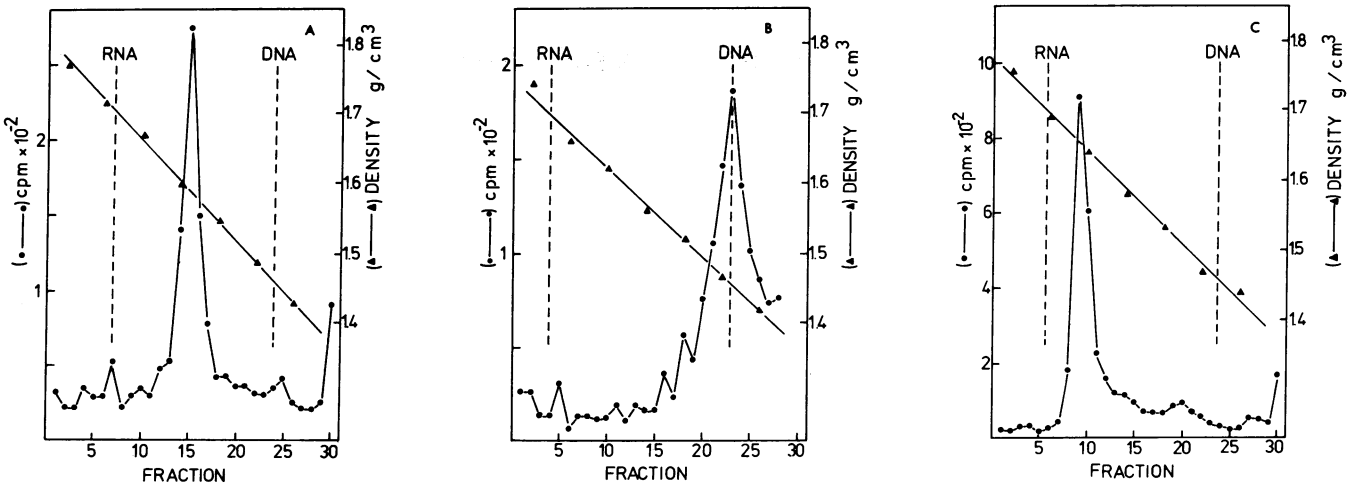

FIG. 2. Cs_2SO_4 density gradient analysis of [^{3}H]DNA, synthesized by purified enzyme from allantoic fluid particles, in response to globin mRNA. Standard polymerase assays, with unlabeled dTTP, dCTP, and dATP and [^{3}H]dGTP were incubated in the presence of 2.5 μg of globin mRNA, 2 μg of $(dT)_{12}$, 5 μg of actinomycin D, and enzyme from particles for 2 hr at 37°. The reaction product was purified by 2-fold phenolization and subsequent alcohol precipitation. It was resuspended in 0.3 M NaCl–0.03 M sodium citrate at pH 7. The density of the product at this stage of treatment is shown in panel A. The product was further treated with 0.25 M KOH for 18 hr at room temperature and then again neutralized. The density of the product at this stage is shown in panel B. The alkali-treated material was incubated in a buffer containing 0.4 M NaCl, 2 mM EDTA, 10 mM Tris·HCl (pH 7.4), and 50% formamide together with 5 μg of globin mRNA, in a total reaction volume of 100 μl, at 37° for 20 hr. The density of the resulting product is shown in panel C. The density gradients were performed by mixing the products with Cs_2SO_4 at a density of 1.55 g/cm^3 and centrifuging this mixture in rotor Ti 50 at 43,000 rpm at 15° for 60 hr. The gradients were fractionated from the bottom, and the density was measured by the refractive index. The fractions then were precipitated with trichloroacetic acid in the presence of bovine serum albumin as carrier, and the radioactivity was measured.

The REV group contains a reverse transcriptase consisting of a single polypeptide with a molecular weight of approximately 80,000 (12). This enzyme sediments in glycerol velocity gradients at the same rate as bovine serum albumin (13). We compared the sedimentation rate of REV enzyme and several markers, in parallel gradients (Fig. 4). The position of the particle enzyme is between those of aldolase and bovine serum albumin, similar to the position of the AMV enzyme, whereas the REV enzyme is found at the position of bovine serum albumin. Another clear distinction of the particle enzyme from the REV enzyme is the preference for different divalent cations. As shown in Table 2, the particle enzyme prefers Mg^{++} over Mn^{++}, whereas the opposite is true for the REV enzyme.

As has been shown (14, 15), the reverse transcriptase from

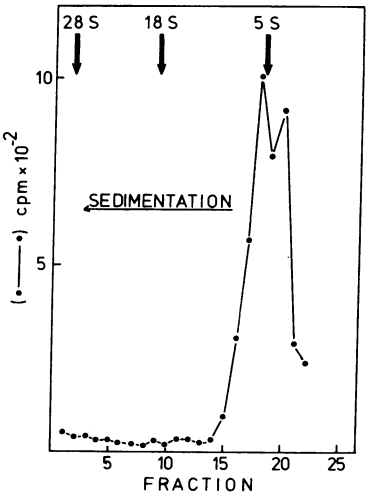

FIG. 3. Material after alkali treatment, as described in Fig. 2B, was layered over a 5–20% sucrose gradient [0.1 M NaCl, 0.01 M Tris·HCl (pH 8.0), 0.001 M EDTA, 0.1% sodium dodecyl sulfate] and centrifuged at 45,000 rpm at 20° for 4 hr. After the centrifugation the gradient was fractionated from the bottom and the trichloroacetic acid-precipitable radioactivity was measured. Arrows indicate the position of marker RNA (ribosomal RNA from chicken myeloblasts) in a parallel gradient, centrifuged under identical conditions. The position of the markers (28S, 18S, 5S) was found by measuring the absorbance.

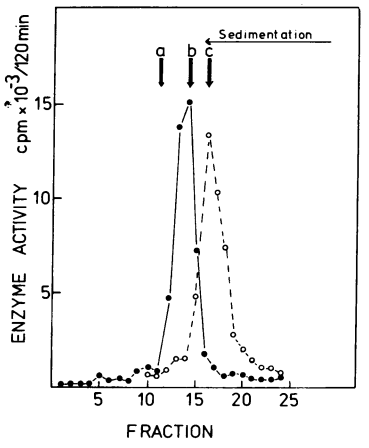

FIG. 4. Velocity sedimentation of reverse transcriptase from particles, REV, and AMV. Purified polymerases were sedimented in glycerol in the presence of 0.4 M KCl, as described by Markus *et al.* (9). The five gradients, centrifuged under identical conditions, contained particle enzyme, REV enzyme, AMV enzyme, aldolase, and bovine serum albumin. After the centrifugation (17 hr) the gradients were fractionated from the bottom. The positions of the polymerases were found by standard polymerase tests with poly(C)·$(dG)_{12}$ as template, the positions of the other marker proteins by measuring the protein concentration. The figure shows activity expressed as cpm of [^{3}H]dGMP incorporated into acid-insoluble material per hr in a reaction volume of 100 μl. (●) Particle polymerase; (○) REV polymerase. (a) Aldolase; (b) AMV polymerase; and (c) bovine serum albumin.

Table 2. Comparison of poly(C)-directed DNA synthesis by reverse transcriptase from particles or REV with different divalent cations

Source of reverse transcriptase	Mg^{++}	Mn^{++}	Relation of activities, Mg^{++}/Mn^{++}
Particles	13,000	1,820	7.15
REV	468,000	1,080,000	0.43

Purified reverse transcriptases were tested under standard conditions in the presence of poly(C)·$(dG)_{12}$ and [^{3}H]dGTP. Tests were performed in the presence of either 10 mM Mg^{++} or 0.4 mM Mn^{++}. Incubation was for 60 min at 37°. The acid-insoluble radioactivity was determined in the total volume.

the ALV/ASV group (subgroups A–E) is a homogeneous immunological species. The reverse transcriptases from this group of viruses, including the spontaneously occurring and the induced endogenous chicken viruses, are very efficiently inhibited by IgG directed against the reverse transcriptase from AMV. Therefore, the IgG neutralization assay is a useful tool for studying the relationship of chicken enzymes to the reverse transcriptases of the ALV/ASV group. Fig. 5 shows the comparative inhibition of particle enzyme and a representative reverse transcriptase from the ALV/ASV group, i.e., AMV reverse transcriptase. In parallel experiments, two different preparations of IgG directed against AMV enzyme were tested for their ability to neutralize the reverse transcriptase activity

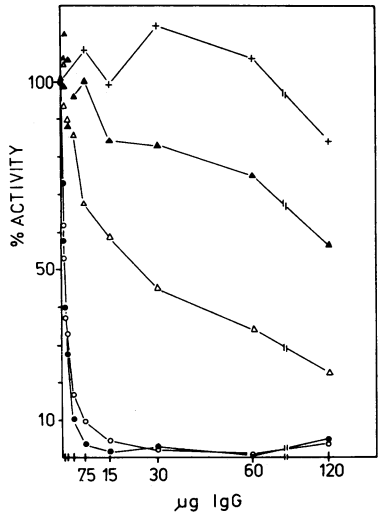

FIG. 5. Comparative inhibition of particle reverse transcriptase and AMV reverse transcriptase by IgG directed against AMV enzyme. Two different IgG preparations directed against AMV reverse transcriptase were serially diluted. Constant amounts of either particle enzyme or AMV enzyme were mixed with identical amounts of each dilution step of both IgG preparations as described by Watson *et al.* (10). The samples were then incubated for 12 hr at 4°. Then each tube was brought to conditions for poly(C)-dependent DNA synthesis, and the residual activity was measured in a 1-hr reaction. Activity (100%) was obtained from tests without added IgG; 100% activity was about 15,000 cpm of [^{3}H]dGMP incorporated per hr in 100 μl of reaction mixture, for particle enzyme and AMV enzyme. Parallel experiments ensured that under conditions of the test the activity was proportional to the amount of enzyme and was linear within the time of the test. (○) Anti-IgG no. 1 + AMV enzyme; (△) anti-IgG no. 1 + particle enzyme; (●) anti-IgG no. 2 + AMV enzyme; (▲) anti-IgG no. 2 + particle enzyme; (+) normal IgG + particle enzyme.

Table 3. IgG necessary for 50% inhibition

IgG directed against reverse transcriptase from	Source of reverse transcriptase tested	μg of IgG necessary for 50% inhibition
AMV (IgG no. 2)	AMV	0.6
	Particles	24.0
AMV (IgG no. 2)	AMV	0.6
	Particles	139.0

The values are obtained from the plot of Fig. 5. The amount of IgG indicated was preincubated with the enzyme in a reaction volume of 50 μl.

of AMV enzyme and particle enzyme. Fig. 5 shows that both preparations give identical and strong inhibition of the reverse transcriptase activity from AMV, but different and very weak inactivation of particle enzyme. From the values of Fig. 5, the amounts of IgG necessary for 50% inhibition are listed in Table 3. As compared to the AMV enzyme, 40 to 200 times more IgG is needed for 50% inhibition of the particle enzyme. The fact that both IgG preparations inhibit AMV polymerase identically, but show different weak inhibition of the particle enzyme, can be interpreted in different ways. One may consider a weak crossreaction with similar determinants in the enzymes or an unrelated reaction as well.

In the meantime, a biochemical comparison of reverse transcriptases from particles and AMV has shown differences in the curves for optimal Mg^{++} and K^+ concentrations and in the K_M values for dGTP (data will be published elsewhere).

Occurrence of the Particle Enzyme. The experiments described so far have been made with enzyme prepared from the allantoic fluid of embryonated eggs of the SPF-VALO flock from Lohmann Tierzucht, Cuxhaven, W.G. We also checked for the presence of enzyme-containing particles in the allantoic fluid of eggs derived from SPAFAS chickens, from a special flock from SPAFAS which are gs^- chf^-, and from a flock of hens from upper Bavaria. In all cases activity with the characteristics shown in Fig. 1 were detected. The activity per ml of allantoic fluid was of the same order of magnitude for all sources tested.

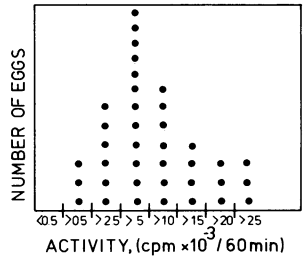

FIG. 6. Particle-bound activity from single eggs. Allantoic fluid of 38 single eggs was taken separately and subjected to low-speed centrifugation to clean it from cells and cell debris. Then 3.2 ml of each single probe were layered over 1 ml of 20% sucrose in a SW 56 tube. The sample was centrifuged for 40 min at 40,000 rpm, and the supernatant and the sucrose were slowly and carefully taken away. Reaction mixture (90 μl), containing 0.1% Nonidet P-40, 2 μg of poly(C)·$(dG)_{12}$, and 5 μCi of [^{3}H]dGTP (8 Ci/mmol), was put into the emptied tube. The bottom, where the nonvisible pellet was expected, was then scratched with the tip of an Eppendorf pipette. The tube was sealed with Parafilm and incubated at 37° for 1 hr; the incorporation was determined. Values given in the figure are corrected for background incorporation, which was obtained from tubes without sedimented allantoic fluid and was about 150 cpm.

To find out whether all or just some eggs used for preparation of enzyme bear the enzyme-containing particles, we prepared crude fractions from 38 single eggs (SPF-VALO) and tested for the presence of poly(C)-dependent poly(dG)-synthesizing activity. In each case the test was positive (Fig. 6).

DISCUSSION

From particles in the allantoic fluid that neither in protein composition nor in biological activity show any relation to the known chicken RNA tumor viruses (i.e., C-type particles), a DNA polymerase, termed particle enzyme, has been isolated. It utilizes synthetic homopolymers and natural RNA with the same relative efficiency as described for viral reverse transcriptases. It is therefore clearly distinct from the chicken cell polymerase α and β (5), from polymerase γ (7), and from terminal deoxynucleotidyltransferase. The purified enzyme could also be clearly differentiated from the reverse transcriptases of REV and ALV/ASV (subgroups A–E), which are the only groups of chicken RNA tumor viruses presently known. The distinction from the REV enzyme is based upon different preference for divalent cations and different sedimentation values in glycerol gradients. Differentiation from the ALV/ASV group has been obtained by application of the IgG neutralization test. As compared to the inactivation of AMV reverse transcriptase by IgG directed against this enzyme, only a weak inactivation of the newly isolated enzyme is observed, which furthermore varies if different IgG preparations are used. As already mentioned, it has been shown (14, 15) that the reverse transcriptases of all tested viruses of the ALV/ASV group (subgroups A–E) are very efficiently and identically inhibited by IgG against AMV reverse transcriptase. It is therefore concluded that the isolated enzyme does not belong to this group of viruses. Since the polymerases of subgroup F and G ALV have not been characterized, we cannot compare the particle enzyme to them. However, these viruses have not so far been found in chicken, and the presence of contaminating viruses of these two subgroups in our preparations can be excluded by the result of the biological tests (see *Materials and Methods*).

Kang and Temin have described a cellular RNA-dependent DNA polymerase activity in a fraction from uninfected chicken cells (8). However the enzyme isolated from this fraction exhibited the template properties of DNA-dependent DNA polymerases (polymerase α or β). One may assume that the purified polymerase described here might be related to the activity in the crude fractions, and that the enzyme finally purified by Temin was not the enzyme responsible for the RNA-dependent DNA synthesis measured in the crude extract. In any case, during the purification procedure used here, an excess of polymerase activity resembling the properties of Temin's purified material was detected.

Recently, Panet *et al.* (16) have mentioned a poly(C)-dependent DNA-polymerizing activity in uninfected chicken cells. This activity may be caused by the particle enzyme and not necessarily by the expression of the polymerase gene of an endogenous virus of the ALV group, as they indicated. In preliminary experiments we could detect particle enzyme-like activity in homogenized chicken embryos as well, but a comparison with the enzyme isolated from the allantoic fluid has still to be made.

On the basis of the data presented here, we cannot exclude the possibility that the particle enzyme originates from an unknown viral agent in chicken. However, the ubiquitous appearance of the enzyme in all flocks tested and its detection in any one of a series of 38 eggs make it a good candidate for physiological reverse transcription as postulated by the provirus theory (4). If this assumption is correct, corresponding enzymes should be detectable also in other species.

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