Properties of Maedi Nucleic Acid and the Presence of Ribonucleic Acid- and Deoxyribonucleic Acid-Dependent Deoxyribonucleic Acid Polymerase in the Virions

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Maedi virus contains a ribonucleic acid (RNA) which can be resolved into three major components, namely, 62S, 33S, and 13S, by sucrose gradient centrifugation. The presence of RNA- and deoxyribonucleic acid (DNA)-dependent DNA polymerase in virions of maedi virus was demonstrated. The enzyme product could be converted into acid-soluble form by pancreatic deoxyribonuclease, but was resistant to digestion by pancreatic ribonuclease and to hydrolysis by NaOH.

Maedi is a slow, progressive lung disease of sheep, causing loss of condition and respiratory distress. The disease usually leads to death within 1 year after the appearance of clinical signs (13). The most pronounced histopathological change in the lungs is a massive proliferation of lymphoreticular cells in the interstitial tissue, which gradually leads to thickening of the interalveolar septa. In advanced cases, the air spaces of the lungs are almost completely replaced by solid lymphoid tissue. The proliferation of lymphoreticular cells is not accompanied by neoplastic changes in the lungs or elsewhere in the host animal.

Maedi virus was first isolated from lungs of sheep showing severe signs of disease (11). A comparative study has shown that the virus is closely related to visna virus (17, 20). However, the growth rate and the appearance of cytopathic effect in sheep cell cultures are markedly slower for maedi virus than for visna virus. Moreover, maedi virus is apparently less stable at low pH and does not show a complete cross-reaction with visna virus in neutralization tests.

Recent biochemical studies have shown that visna virus contains a ribonucleic acid (RNA)- and deoxyribonucleic acid (DNA)-dependent DNA polymerase and an RNA of high molecular weight (4, 6, 7, 10, 14). The aim of the present study was to obtain information about the nature of the maedi virus nucleic acid and to determine whether the virion contains DNA polymerase of the same type as those demonstrated in visna virus. This information will be useful in our projected biochemical study of visna virus-host cell

interactions, because a comparative study of maedi virus may shed some light on the biochemical mechanism causing the difference in growth rate and cytopathogenicity of the two viruses. This report presents evidence for the presence of DNA polymerase in virions of maedi virus and shows that the virus contains RNA similar to that of visna and tumor viruses.

MATERIALS AND METHODS

Chemicals. Uridine-5-³H and ³H-methyl-thymidine triphosphate (³H-TTP) were purchased from Schwarz/Mann, Orangeburg, N.Y. ¹⁴C-methyl-thymidine was a product of New England Nuclear Corp., Boston, Mass. Nonlabeled deoxyribonucleoside triphosphates were supplied by Sigma Chemical Co., St. Louis, Mo. The 23S ribosomal RNA of *Escherichia coli* was the product of Miles Laboratories, Kankakee, Ill. The electrophoretically purified bovine pancreatic deoxyribonuclease was obtained from Worthington Biochemical Corp., Freehold, N.J. Pancreatic ribonuclease was purchased from Calbiochem, Los Angeles, Calif.

Cell cultures and media. Cultures of sheep choroid plexus (SCP) cells were used for virus production. The method of cell culture has been described (7).

Infection of cells and preparation of radioactive virus. The cells grown in 250-ml plastic flasks (Falcon Plastics) were inoculated with maedi virus (strain M88) at a multiplicity of about one TCID₅₀ per cell. The inoculated cultures were incubated at 37 C for 3 hr in 10 ml of maintenance medium containing 10 μCi of ³H-uridine (25 Ci/mmmole) per ml or 10 μCi of ³H-uridine plus 1 μCi of ¹⁴C-methyl-thymidine (20 to 30 mCi/mmmole) per ml. Thereafter, 30 ml more of the same medium was added to the cultures which were incubated at 37 C. The infectious fluid was harvested

14 days after viral inoculation when marked cytopathic effect was observed. The virus preparation will be referred to as labeling virus.

For experiments designed to study the effect of long incubation time on the integrity of viral nucleic acid, the infected cultures were incubated with the medium containing no radioactive isotopes for 48 hr. The cold medium was then replaced with 20 ml of fresh medium containing radioactive precursor of nucleic acid as described above. Thereafter, the medium was changed every 24 hr. The infectious fluid was pooled and stored at 4 C. The concentration and purification of virus commenced at the third change of medium. This means that the longest time for storing the infectious fluid at 4 C was 3 days. This virus preparation will be referred to as short labeling virus.

Measurement of radioactivity, purification of virus, and extraction of viral RNA. The methods used have been described previously (7).

Assay of DNA polymerase. The assay conditions have been described elsewhere (6).

Analysis of nucleic acid by sucrose gradient centrifugation. The method described for visna RNA analysis was employed (7).

RESULTS

Analysis of nucleic acid isolated from maedi virus. A ^3H -labeled nucleic acid was extracted from purified maedi virus by the phenol-sodium dodecyl sulfate (SDS) method. The nucleic acid was centrifuged on a sucrose gradient containing 0.1 M NaCl and 1 mM disodium ethylenediaminetetraacetate (EDTA). The profile of the acid-precipitable radioactivity from long labeling virus is illustrated in Fig. 1A. It can be seen that the nucleic acid extract contained three major components sedimenting at 62S, 33S, and 13S, respectively, with reference to the *E. coli* 23S ribosomal RNA. The 62S component accounted for about 15% of the total radioactivity, and the 33S component constituted about 10% of the total nucleic acid. The remaining 75% of the maedi nucleic acid was sedimented around the area of 13S. When nucleic acid from long labeling virus grown in the presence of ^3H -uridine and ^{14}C -methyl-thymidine was analyzed by sucrose gradient centrifugation, the same profile of ^3H radioactivity was observed. However, ^{14}C radioactivity was not detectable. Under the conditions employed, an incorporation of 0.1 pmole of ^{14}C -thymidine would have been detected.

The appearance of the 13S component in the viral nucleic acid probably represents a degraded molecule in the virions resulting from the long incubation (14 days) of the virus at 37 C. To test this possibility, it was desirable to harvest the virus soon after it was released into the medium, by employing the procedures of Bader and Steck (2). However, because of the slow growth of maedi virus, the shortest time to obtain detectable

FIG. 1. Sucrose gradient centrifugation of maedi virus nucleic acid. (A) ^3H -uridine-labeled maedi virus was harvested 14 days after inoculation and was purified by potassium tartrate gradient. The nucleic acid was extracted by the phenol-SDS method (7). The viral extract plus 5 optical density units of 23S *E. coli* ribosomal RNA was centrifuged on a 5 to 20% sucrose gradient in a Beckman SW65 rotor at 257,000 $\times g$ for 45 min at 4 C. Samples (0.2 ml) were collected from the bottom of the centrifuge tube. To each fraction, water was added to 1 ml, and the absorbance at 260 nm was measured in a Gilford spectrophotometer. A 0.1-ml amount of yeast RNA (0.1 mg) and a 1-ml amount of 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate were added to each fraction in that order. After chilling in ice water for 10 min, the acid-precipitable nucleic acid was collected on a Gelman glass-fiber filter (type E) and washed three times with 10 ml of cold 5% trichloroacetic acid. The filter was dried on a hot plate and placed in a glass scintillation vial containing 10 ml of omnifluor (New England Nuclear Corp.) in toluene. The radioactivity was measured in a Picker Nuclear Liquimat 330 scintillation counter with an efficiency of 45% for ^3H . (B) The nucleic acid was extracted from ^3H -uridine-labeled maedi virus which was collected from tissue cultures at 24-hr intervals. The analysis of the nucleic acid was carried out by the procedures described in A.

virus in the medium was about 24 hr. Accordingly, ^3H -nucleic acid was isolated from virus released into the medium for no longer than 24 hr. The radioactive profile of the acid-precipitable nucleic

acid from the short labeling virus is shown in Fig. 1B. It can be seen that the 13S component was still present in the extract but represented about 28% of the total radioactivity, as compared with 75% for the 13S component shown in Fig. 1A. It is also shown that the 33S was the predominant component (32%) when virus was harvested in shorter intervals of incubation at 37 C.

To characterize further the viral nucleic acid, it was incubated with ribonuclease and deoxyribonuclease. The results of these experiments (Fig. 2) show that ^3H -uridine-labeled nucleic acid was almost completely digested by pancreatic ribonuclease. On the other hand, the deoxyribonuclease-treated sample showed a profile similar to that of the untreated sample (see Fig. 1). The data demonstrate that an RNA is present in maedi virus.

Presence of RNA- and DNA-dependent DNA polymerase in maedi virus. Table 1 shows that

FIG. 2. Sucrose gradient centrifugation of maedi nucleic acid labeled with ^3H -uridine. The nucleic acid was extracted from purified virus by the phenol-SDS method, and was coprecipitated with 1 mg of yeast RNA by ethanol. It was dissolved in 1 ml of tris(hydroxymethyl)aminoethane-hydrochloride (0.01 M, pH 7.4) containing 0.005 M MgCl_2 (TM buffer). The solution was divided into two equal volumes, and these samples were incubated, with 50 μg of ribonuclease or deoxyribonuclease per ml at 37 C for 30 min. After chilling in ice water, NaCl was added to each sample to 0.1 M. The treated samples were then analyzed by sucrose gradient centrifugation according to the method described in Fig. 1.

TABLE 1. Template requirement of DNA polymerase^a

Treatment	Thymidine monophosphate incorporated (pmoles)	Percentage of untreated
Untreated.....	22.70	100
Ribonuclease (300 $\mu\text{g}/\text{ml}$)..	6.10	27
Ribonuclease (300 $\mu\text{g}/\text{ml}$), 10 μg of activated calf thymus DNA added ^b	42.50	190
Zero time sample.....	0.18	0.8

The purified virions were incubated with 0.2% Nonidet P-40 (Shell) at room temperature for 15 min. Ribonuclease, which was dissolved in water (1 mg/ml) and had been boiled for 10 min, was added to the suspension of the disrupted virions, and the mixed suspension was incubated for an additional 30 min at the same temperature. Water was substituted for the ribonuclease solution in the untreated sample. A 0.1-ml sample of the solution containing 12 μg of viral protein [measured by the method of Lowry et al. (8), with bovine serum albumin as standard] was then added to 0.1 ml of a reaction mixture which contained 0.05 μmole each of 2'-deoxyadenosine 5'-triphosphate, 2'-deoxycytosine 5'-triphosphate, and 2'-deoxyguanosine 5'-triphosphate and 0.0003 μmole of ^3H -TTP (1,154 counts per min per pmole), 15 μmoles of tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.3), 1.2 μmoles of MgCl_2 , 12 μmoles of NaCl, and 4 μmoles of 2-mercaptoethanol. The mixture was incubated at 37 C for 1 hr; then 0.1 ml of yeast RNA (0.1 mg) and 0.3 ml of 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate were added to the reaction mixture. After chilling in ice water for 10 min, the mixture was filtered through a Gelman glass-fiber filter (type E) and washed three times with 10 ml of 5% trichloroacetic acid. The radioactivity was measured by the procedures described in Fig. 1. The zero time sample was a duplicate of the untreated reaction mixture.

^b Activated calf thymus DNA was prepared according to the method of Aposhian and Kornberg (1).

maedi virus is capable of synthesizing DNA under the direction of resident RNA. The activity was reduced to 27% when the disrupted virions were treated with ribonuclease. On the other hand, the enzymatic activity of the ribonuclease-treated reaction mixture was greatly stimulated by addition of activated calf thymus DNA. The data indicate that the DNA-synthesizing system in maedi virus can be directed by either viral RNA or exogenous DNA. The requirements of the DNA polymerase activity are presented in Table 2. All four deoxyribonucleoside triphosphates were required for maximal activity. In the absence

TABLE 2. Requirements for enzyme activity^a

Reaction system	Thymidine monophosphate incorporated (pmoles)
Complete.....	9.83
Minus dATP, dCTP, and dGTP....	0.25
Minus dATP.....	0.50
Minus 2-mercaptoethanol.....	0.56
Minus MgCl ₂	4.70
Minus Nonidet P-40.....	0.48
Minus virus.....	0.25

^a The assay conditions were the same as those described in Table 1. Each reaction mixture contained 5 μ g of viral protein. The specific radioactivity of ³H-TTP was 630 counts per min per pmole. dATP = 2'-deoxyadenosine 5'-triphosphate; dCTP = 2'-deoxycytosine 5'-triphosphate; dGTP = 2'-deoxyguanosine 5'-triphosphate.

of three deoxyribonucleoside triphosphates, there was no incorporation of thymidine monophosphate (TMP). Omission of deoxyadenosine triphosphate in the reaction mixture reduced the incorporation of TMP to 2.5%. To attain full enzymatic activity, the virions must be disrupted by a nonionic detergent such as Nonidet P-40. A sulfhydryl agent such as 2-mercaptoethanol was essential for maximal activity. A 50% reduction of TMP incorporation was observed when MgCl₂ was deleted from the reaction mixture, suggesting a contamination of divalent cations in the reaction mixture. The data indicate that TMP was incorporated into a heteropolymer by the DNA-synthesizing system in maedi virus.

To demonstrate further an enzymatic role in the incorporation of TMP, a kinetics experiment was performed. The results of this experiment (Fig. 3) show that the rate of reaction was linear in the first 60 min. Thereafter, the rate of incorporation leveled off.

Nature of the enzyme product. Table 3 shows the properties of the enzyme product isolated from a reaction mixture by phenol-SDS extraction and ethanol precipitation. It can be seen that the product was fully resistant to ribonuclease. About 91% of the product remained acid-precipitable after treatment with NaOH. On the other hand, deoxyribonuclease converted 97% of the product into acid-soluble form. The results of this experiment show that the enzyme product is DNA.

Correlation of enzyme activity with viral infectivity. Figure 4A shows the correlation of enzyme activity and viral infectivity in a potassium tartrate gradient. High enzyme activity and viral infectivity were located at a density of 1.16 g/ml.

FIG. 3. Kinetics of DNA polymerase activity. The volume of the reaction mixture was five times that of the mixture described in Table 1. Samples (0.1 ml) were withdrawn from the reaction tube at the time intervals indicated, and the analysis was carried out according to the procedures described in Table 1. Specific radioactivity of ³H-TTP was 750 counts per min per pmole.

TABLE 3. Property of enzyme product^a

Treatment	Acid-precipitable radioactivity (counts/min)	Percentage of untreated
Untreated.....	29,500	100
Ribonuclease (100 μ g/ml).....	31,210	106
Deoxyribonuclease (100 μ g/ml).....	958	3
NaOH.....	26,811	91

^a The volume of the reaction mixture was 10 times that of the reaction mixture described in Table 1 and contained 250 μ g of viral protein. The reaction was allowed to take place for 2 hr at 37 C; then 1 mg of yeast RNA was mixed with the reaction mixture, and the nucleic acid was extracted by the phenol-SDS method as previously described (7). The nucleic acids were dissolved in 1 ml of TM buffer, and the solution was divided into four equal volumes. The untreated samples and the ribonuclease- and deoxyribonuclease-treated samples were incubated at 37 C for 30 min, whereas the treatment with NaOH was carried out according to the method of Baltimore (3). The acid-precipitable product was analyzed by the method described in Fig. 1.

Another peak, banded at a density of 1.23 g/ml (fraction 11) also exhibited both enzyme and viral activities. The latter peak contained cell debris to which a substantial amount of virions was attached. Significant enzyme activity was observed near the top of the gradient (fraction 30)

FIG. 4. Enzyme activity and viral infectivity in fractions of potassium tartrate gradient. (A) Correlation between endogenous enzyme activity and viral infectivity. The virus was obtained from tissue culture fluid 14 days after inoculation. The fractionation of the gradient was performed by an ISCO model D gradient fractionator, and the samples were collected from the top of the gradient as described previously (7). Samples (0.1 ml) were withdrawn from each fraction for enzyme assay and for viral infectivity titration (16). For enzyme assay, potassium tartrate was removed by ultracentrifugation as described previously (6). The assay procedures and analysis of the enzyme product were the same as those described in Table 1. The fraction was numbered from bottom (left) to top (right). (B) Correlation between endogenous activity and exogenous activity of DNA polymerase. The virus was harvested 2 days after inoculation. The viral purification and enzyme assay were the same as those described in A.

where viral infectivity was not detectable. Figure 4B shows the enzyme activity directed by endogenous and exogenous templates in another potassium tartrate gradient. The endogenous activity completely overlapped the exogenous activity, suggesting that the enzyme detected in the three peaks was derived from the virions.

DISCUSSION

Maedi virus contains an RNA which can be resolved into three major components sedimenting at 62S, 33S, and 13S. The maedi RNA is similar to the RNA extracted from visna virus

(4, 7) and progressive pneumonia virus (PPV) (14) with respect to the presence of a 62S component. However, in every nucleic acid preparation from the long labeling maedi virus, the RNA contains primarily the 13S component. Furthermore, the 33S component of maedi RNA always represents a significant or even predominant proportion of the total RNA under the conditions of the RNA extraction. These two latter properties were also observed by Stone et al. (14) for PPV and by Jarret et al. (5) for feline leukemia virus. The appearance of the 13S component and the predominance of the 33S RNA in the short labeling maedi virus are, on the other hand, in marked contrast to the visna RNA, which was normally composed of the 60 to 70S and 4S components, even though visna virus was routinely harvested 3 to 4 days after inoculation (7). The quantitative difference of the 13S component in the long and short labeling preparations of maedi virus suggests that this 13S RNA is a degraded product of the heavier molecules. Bader and Steck (2) have demonstrated that leukemia viral RNA can be converted into smaller components in the virions by incubating the virus at 37 C. These authors found that the half-life of the native viral RNA was about 2 hr. The fact that the smaller components of leukemia viral RNA appear only after physical or chemical treatment of the native viral RNA led Bader and Steck (2) to suggest the existence of a noncovalent bonding as a secondary structure in the leukemia viral RNA. In contrast, the 13S and 33S components in the maedi nucleic acid are always observed without any special treatment. It appears that the secondary structure, if any, of the maedi nucleic acid is very weak and is unable to maintain a stable stage of the molecule.

Another unique feature of maedi virus lies in the consistent presence of a noninfectious low-density material with high DNA polymerase activity (Fig. 4). In the two experiments depicted in Fig. 4, only the pellet resulting from centrifugation at $75,000 \times g$ for 1 hr was assayed for enzyme activity. Thus, the DNA polymerase activity detected near the top of the gradient probably represents a nucleoprotein released from disrupted virions during viral purification. It should be pointed out that the results shown in Fig. 4A were obtained from the virus harvested 14 days after inoculation, whereas those in Fig. 4B represent the virus harvested 2 days post-inoculation. It is noted that the low-density enzyme activity was about the same in both experiments. This observation suggests that a substantial amount of noninfectious disintegrated virus already exists in the tissue culture fluid soon after the beginning of viral release into the

medium at approximately 30 hr after inoculation (17). In contrast, the light-density nucleoprotein was not observed in visna virus (6), even though visna virus was harvested 4 days postinoculation—2 days later than the maedi virus used in the experiment shown in Fig. 4B. Maedi virus particles seem, therefore, to be more labile than visna virus.

With the exceptions pointed out above, the data presented in this paper show that maedi virus is similar to visna virus and PPV and to RNA tumor viruses in the biochemical properties studied (9, 10). Thus, in addition to the properties previously found to be shared by the visna-maedi virus group and RNA tumor viruses (18), all of these viruses have now been shown to contain 60 to 70S RNA and reverse transcriptase. The visna-maedi virus group and the RNA tumor viruses are similar not only with respect to a variety of physical, biochemical, and biological properties studied in cell cultures, but also to a certain extent in their relationship to the animal host (19). All of these viruses cause a slow infection to their hosts, as first pointed out by Sigurdsson (12). Virus multiplication usually continues for a long time in the infected animals before clinical signs appear, and often in the presence of neutralizing antibodies which do not protect the animals against disease. All of the viruses cause a massive proliferation of certain types of cells in the host animal rather than cell degeneration and cell death. The significant difference lies in the apparent absence of malignant, neoplastic changes in visna and maedi.

A number of questions arise in connection with the apparent similarities and dissimilarities of visna-maedi viruses and RNA tumor viruses. First, are the similarities in the pathogenesis of the slow progressive diseases they cause in animals related to the similarities in their biochemical properties? Second, does the lack of tumorigenicity in visna and maedi reflect a biochemical difference between visna-maedi viruses and tumor viruses? If so, is this difference expressed in the virus-host relationship at the cellular level? To answer these questions, it is necessary to study in great detail (i) the nucleic acids of the two groups of viruses and the products of the viral genomes and (ii) the capability of visna-maedi viruses to cause malignant transformation of cells in vitro. Transformation of murine cells by visna virus and PPV has been reported by Takemoto and Stone (15). In view of the important implications of such an observation, it seems to be imperative that the study of Takemoto and Stone be confirmed and extended to other cell systems. Maedi

virus might be better suited than visna virus for this type of study in sheep cells, because it is less cytopathogenic.

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