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Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerase Activity in Cells Infected with Influenza Virus

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Deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase activity was assayed on nuclear preparations of chick embryo fibroblast cells at various times after infection with an influenza A virus (fowl plague virus) and was compared with the activity of uninfected cells. Polymerase activity was increased by about 60% by 2 hr after infection, and this increase coincided with an increase in RNA synthesis in infected cells, as determined by pulse-labeling with uridine. No differences could be detected between the polymerases of infected and uninfected cells as to their requirements for DNA primer, divalent cations, and nucleoside triphosphates, and they were equally sensitive to addition of actinomycin D to the reaction mixture. It is possible that host cell DNA-dependent RNA polymerase is involved in the replication of influenza virus RNA.

There is a considerable body of information concerning the synthesis of virus-specific ribonucleic acid (RNA) in cells infected with small RNA viruses and RNA phages (18). Of the animal viruses, poliovirus and mengovirus have been particularly well studied, and it has been established that these viruses multiply within their host cells by inducing the formation of a virus-specific RNA synthesizing system in the cytoplasm, which is independent of the normal cell RNA synthesizing mechanism (20). In addition, it has been shown that the host cell nuclear deoxyribonucleic acid (DNA)-dependent RNA polymerase is inhibited shortly after infection with these viruses (2, 14), and replaced by a new cytoplasmic RNA-dependent RNA polymerase (3). Thus, small viruses of this type are capable of multiplying in cells in which all cellular RNA synthesis has been suppressed by treatment with actinomycin D (22), a potent inhibitor of DNA-dependent RNA polymerase (11). With influenza viruses, however, replication is inhibited by doses of actinomycin D so low that secondary effects of the drug are ruled out (4). Moreover, synthesis of new DNA is not required during multiplication (24). These findings, coupled with other aspects of influenza virus replication, such as its extreme sensitivity to ultraviolet light (4), and the fact that replication of the viral RNA takes place within the nucleus rather than the cytoplasm of the host cell (5, 25), suggest that a DNA-directed event may be involved in the multiplication of influenza viruses.

As a first step in investigating this aspect of influenza RNA replication, we have examined the activity of host-cell DNA-dependent RNA polymerase at various stages of infection of chick embryo fibroblast cells with fowl plague virus (FPV), an influenza A strain. This virus is particularly suitable for study as it multiplies rapidly and to a high titer in chick fibroblasts.

MATERIALS AND METHODS

Virus stocks. The Rostock strain of FPV was used throughout this study. Virus stocks were prepared by allantoic inoculation of 10-day fertile eggs with 0.02 ml of infected allantoic fluid containing approximately 1,000 egg infectious doses of FPV. After 30 hr of incubation at 37 C, the allantoic fluid was harvested and clarified by centrifugation at 5,000 $\times$ *g* for 10 min. The supernatant fluid was centrifuged at 30,000 $\times$ *g* for 1.5 hr. The resultant pellet was resuspended in phosphate-buffered saline (PBS) containing 0.1% gelatin to give a titer of approximately 5,000 agglutinating doses per ml and was stored in sealed glass ampoules at -70 C. Such virus preparations had an infective titer of 2×10^5 plaque-forming units (PFU) per agglutinating dose.

Cell cultures and infection. Cell cultures were prepared from 10-day-old chick embryos after trypsinization and growth in tissue culture medium 199 containing 10% calf serum. Confluent cell sheets had formed after 48 hr of incubation at 37 C in an atmosphere of 5% CO_2 in air.

The monolayer cultures, in plastic petri dishes

(6 or 10 cm in diameter; Falcon Plastics, Los Angeles, Calif.), were infected as follows. The cells were washed once with warm sterile PBS, and then were exposed to FPV at a multiplicity of 1 to 2 PFU/cell in a volume of 0.5 or 1.0 ml for 6- and 10-cm dishes, respectively. Adsorption was allowed to proceed for 30 min at room temperature. Control cultures were handled in a similar manner but received only PBS. The supernatant fluid was removed, fresh growth medium (at 37 C) was added to each dish, and the dishes were incubated at 37 C. Zero time corresponded to the time of addition of fresh growth medium.

Virus infectivity titration. FPV was titrated by plaque assay on 48-hr primary chick embryo cell monolayers grown in 6-cm plastic petri dishes. The diluent was PBS containing 0.1% gelatin. The cells were washed with PBS, and duplicate monolayers were inoculated with 0.2 ml of successive 10-fold dilutions of virus. After adsorption for 1 hr at room temperature, the inocula were poured off, and 4 ml of nutrient agar overlay (medium 199 containing 5% calf serum and 1% agar) was added to each dish. After incubation for 48 hr at 37 C in an atmosphere of 5% CO_2 in air, 2 ml of indicator medium (0.9% agar containing 0.02% neutral red) was added to each dish, and the plaques were counted after a further 2 hr at room temperature.

Hemagglutination (HA) titration. Serial twofold dilutions of FPV in a volume of 0.25 ml of PBS were prepared in plastic hemagglutination trays. An 0.25-ml amount of a 1% suspension of chicken red blood cells in PBS was added to each cup, and the HA titer was read after 45 min at room temperature. The titer was expressed as the reciprocal of the highest dilution of virus giving minimal but definite agglutination.

Virus growth. Virus growth was measured as follows. At various time intervals postadsorption, all of the medium except 1 ml was removed from two petri dishes, and the dishes were stored at −70 C. Cells and medium were frozen and thawed three times and removed from the dish. These preparations were centrifuged at 1,000 × *g* for 5 min; the supernatant fluids were used for HA titrations and plaque assays.

RNA synthesis. Cells were pulse-labeled by the addition of uridine-5-^3H (2 μc per 6-cm petri dish culture) for 15 min at various times after infection. Addition of the labeled precursor was carried out in a room at 37 C, to minimize disturbances due to temperature changes. After 15 min, the medium was decanted, and 2 ml of ice-cold 5% trichloroacetic acid was added to each culture. After removing portions for determining the radioactivity in the trichloroacetic acid-soluble nucleotide pool, the acid-insoluble material was washed three times with 2 ml of ice-cold 5% trichloroacetic acid and finally was heated in 2 ml of 5% trichloroacetic acid for 1 hr at 90 C. Samples of the hydrolysate were removed for RNA determination and measurement of radioactivity. A 0.5-ml sample was dissolved in 10 ml of dioxane scintillator (19) and counted in a Packard Tri-Carb scintillation counter with an efficiency for tritium of 38%. The specific activity of infected cultures was expressed as a percentage of that of uninfected cultures.

Cell fractionation. Cell fractions containing polymerase activity were prepared as follows. Culture fluids were poured off, and the monolayers were washed twice in ice-cold sucrose-$MgCl_2$ solution [0.25 M sucrose, 0.001 M $MgCl_2$ in tris(hydroxymethyl)aminomethane (Tris) chloride buffer at *p*H 8.0]. To each dish, 2 ml of cold sucrose-$MgCl_2$ plus 0.5 ml of ethylenediaminetetraacetate (1:5,000) were then added, and all subsequent procedures carried out at 2 C.

The cells were removed from the dish by scraping with a silicone-rubber policeman and centrifuged at 400 × *g* for 10 min. The cell pellet was resuspended in 5 ml of sucrose-$MgCl_2$. Cells were disrupted by five strokes of a tight-fitting Dounce homogenizer. Preparations examined by phase-contrast microscopy showed that over 90% of the cells were disrupted by this procedure. The suspension of disrupted cells was centrifuged at 600 × *g* for 10 min, and the resulting pellet containing nuclei was resuspended in sucrose-$MgCl_2$ solution. This fraction was used to determine DNA-dependent RNA polymerase activity. Microsomal fractions were prepared from the supernatant fluid by centrifugation at 105,000 × *g* for 1 hr in a Spinco ultracentrifuge. The pellet was resuspended in sucrose-$MgCl_2$ solution and used for the assay of RNA-dependent RNA polymerase.

DNA-dependent RNA polymerase. DNA-dependent RNA polymerase activity was assayed by a procedure based on that described by Goldberg (10). The reaction mixture contained 35 μmoles of Tris chloride (*p*H 8.1), 0.8 μmoles of $MgCl_2$, 5 μmoles of $MnCl_2$, 7 μmoles of 2-mercaptoethanol, 5 μmoles of phosphoenolpyruvate (PEP), 10 μg of pyruvate kinase, 50 μg of calf thymus DNA, 0.1 ml of saturated ammonium sulfate, 50 mμmoles each of the 5′-triphosphates of adenine (ATP), cytidine (CTP), and uridine (UTP), 50 mμmoles of ^3H-guanosine-5-triphosphate (^3H-GTP), specific activity 25 μc/μmole, and 0.1 ml of enzyme suspension (0.2 to 0.6 mg of protein) in a total volume of 0.6 ml. Incubation was carried out at 37 C for 30 min. All assays were duplicated and corrected for radioactivity incorporation into identical reaction mixtures, which were kept at 2 C for 30 min.

The reaction was stopped by placing the tubes at 2 C and adding 1 ml of ice-cold 0.5 N perchloric acid containing 0.125 M sodium pyrophosphate.

To each tube was added 0.1 ml of carrier (5 mg of yeast RNA plus 5 mg of bovine serum albumin per ml). The precipitate was treated with sodium hydroxide, sodium pyrophosphate, perchloric acid, and trichloroacetic acid; dissolved in 10 ml of dioxane scintillator as described by Martin and Sonnabend (19); and counted in a Packard Tri-Carb scintillation counter with an efficiency for tritium of 38%. RNA polymerase activity was expressed as counts per minute incorporated into an acid-insoluble product during 30 min of incubation at 37 C.

RNA-dependent RNA polymerase. The activity of this enzyme was assayed as described by Martin and Sonnabend (19), except that incubation was for 30 min at 37 C. Washing of the acid-insoluble ma-

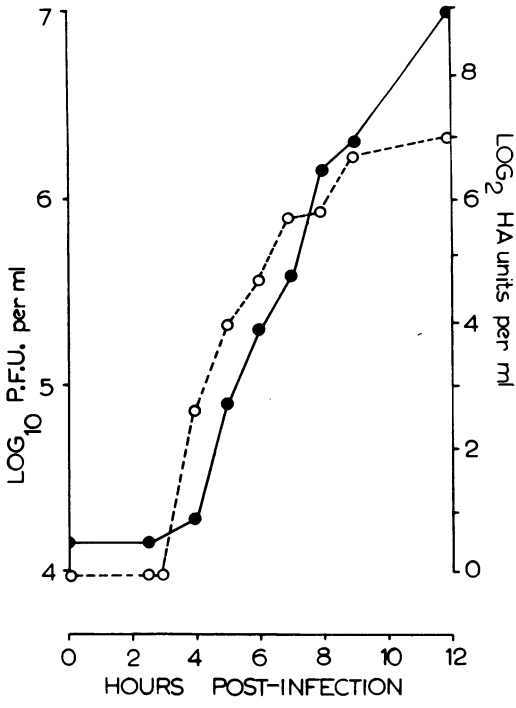

FIG. 1. *Growth of FPV in chick embryo fibroblast monolayer cultures. Total virus production was measured as described in Materials and Methods. Symbols:* ○, *total hemagglutinin;* ●, *total infectious virus (PFU).*

terial and radioactivity determinations were as described for DNA-dependent RNA polymerase.

RNA determination. An orcinol method was used to determine RNA. An 0.5-ml sample was diluted with 0.5 ml of water before addition of 1.5 ml of 0.1% ferric chloride in 12 N HCl containing 10% alcoholic orcinol (100 mg of orcinol per ml of 95% ethyl alcohol). The mixture was incubated in a boiling-water bath for 30 min and then cooled, and the optical density was determined at 670 mμ. Yeast RNA was used as the standard.

Protein determination. The method of Lowry et al. (17) was used for protein determination. The test was standardized with human serum which was in turn standardized by the biuret method (12).

Reagents. Uridine-5-^3H (5.0 c/mmole) was purchased from the Radiochemical Centre, Amersham, Bucks, England. Guanosine-5'-triphosphate-^3H (1.0 c/mmole) was purchased from Schwarz Bio Research Inc., Orangeburg, N.Y. Unlabeled nucleoside triphosphates, pyruvate kinase (crystalline, from rabbit muscle), trisodium PEP, 2-mercaptoethanol, and calf thymus DNA (type 1) were purchased from Sigma, London Chemical Co Ltd., London, England. Pancreatic ribonuclease, three times crystallized, and deoxyribonuclease I, electrophoretically purified (free of ribonuclease), were purchased from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Growth of FPV in chick embryo fibroblasts. The replication of FPV was studied by measuring total hemagglutinin and infectious virus at various times after infection (Fig. 1). Hemagglutinin production was first detected in the cells between 3 and 4 hr postinfection and was followed by the appearance of new mature virus at 4 hr, detected by plaque titration. The maximal rate of virus production occurred 4 to 8 hr postinfection.

Synthesis of RNA in infected cells. RNA synthesis was measured in both virus-infected and control cell cultures. However, since even low levels of actinomycin D inhibit FPV production (4), it was not possible to study viral RNA synthesis in the absence of cellular RNA synthesis, as has been done, for example, with reovirus (27). Accordingly, the net rate of RNA synthesis was determined in virus-infected cells and expressed as a ratio, compared with that of uninfected cells (Fig. 2).

A small but consistently observed early peak of RNA synthesis occurred 30 min after infection, and this was followed by a second period of increased RNA synthesis between 2 and 4 hr after infection. Associated with the latter peak of RNA synthesis there was an increased labeling in the acid-soluble pool, which agrees with a recent report by Scholtissek et al. (23).

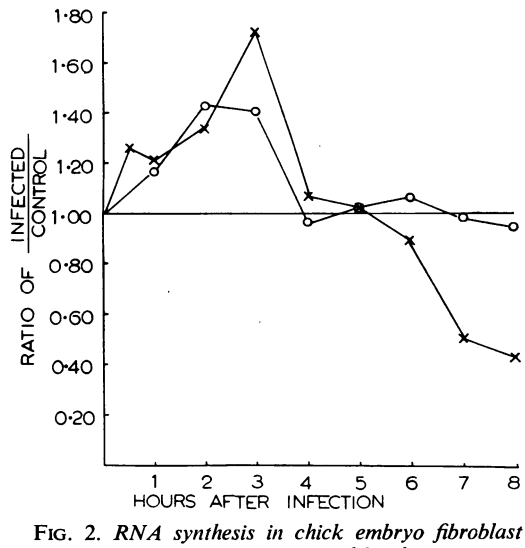

FIG. 2. *RNA synthesis in chick embryo fibroblast cells infected with FPV, as measured by the incorporation of ^3H-uridine during a 15-min period prior to the time-point shown on the graph. All values are the means of triplicate determinations and are expressed as a percentage of those of uninfected cells. Symbols:* ○, *trichloroacetic acid-soluble radioactivity;* ×, *specific activity of RNA.*

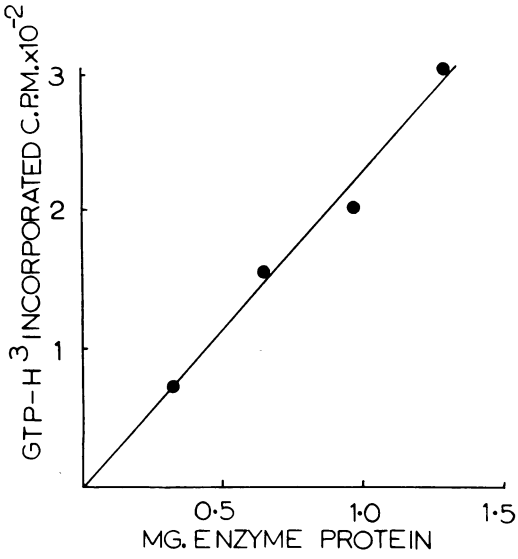

FIG. 3. *Dependence of DNA-dependent RNA polymerase activity on amount of enzyme preparation added to the reaction mixture. Various quantities of a nuclear suspension prepared from chick fibroblast cells were incubated for 30 min at 37 C with* ^{3}*H-GTP, ATP, CTP, UTP, PEP, pyruvate kinase, DNA,* $(NH_4)_2SO_4$, $MgCl_2$, $MnCl_2$, *2-mercaptoethanol, and Tris buffer (pH 8.1) in a total volume of 0.6 ml, and the acid-insoluble radioactivity was measured as described in Materials and Methods.*

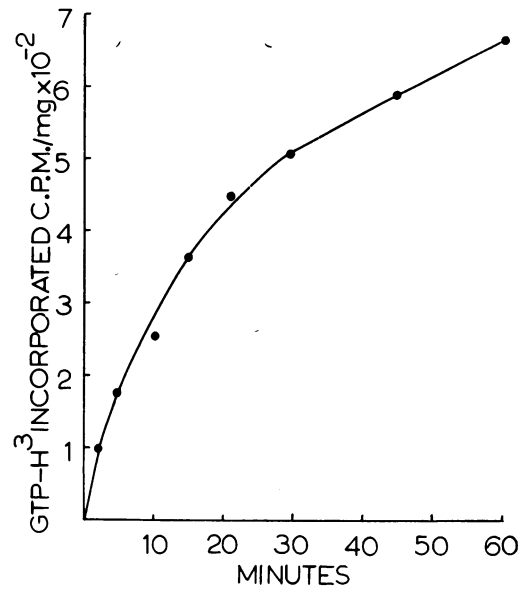

FIG. 4. *Time course of incorporation of* ^{3}*H-GTP into an acid-insoluble product. A 1.0-ml amount of a nuclear suspension from chick embryo fibroblast cells was incubated at 37 C with 8 ml of a reaction mixture, as described in the legend to Fig. 3. At intervals, duplicate 0.5-ml samples of the mixture were removed and analyzed for acid-insoluble radioactivity, as described in Materials and Methods. Values were corrected for incorporation into identical samples incubated at 2 C for the same period of time.*

The coincidence of this second peak of RNA synthesis with the period of viral RNA formation, as demonstrated by oligonucleotide analysis (24), strongly suggests that it represents the synthesis of viral RNA.

Synthesis of RNA in virus-infected cells did not fall below control level until 5 to 6 hr post-infection, i.e., during the period of mature virus release. This contrasts with the inhibition of cellular RNA synthesis which is observed before viral RNA replication in cells infected with picornaviruses (18) and vesicular stomatitis virus (15).

DNA-dependent RNA polymerase activity. The nuclear fraction from chick embryo fibroblast cells was incubated with ^{3}H-GTP by use of the assay conditions described above. Incorporation of ^{3}H-GTP into an acid-insoluble product was directly proportional to the amount of enzyme added to the reaction mixture (Fig. 3). The reaction proceeded rapidly for the first 20 min of incubation, and more slowly thereafter (Fig. 4).

Enzyme assays carried out on cells 2 hr after infection with FPV showed that DNA-dependent RNA polymerase activity was increased by 50% in the nuclear fraction of the cells. No appreciable activity was found in the mitochondrial, micro-

TABLE 1. *Requirements of DNA-dependent RNA polymerase from chick embryo fibroblast cells, uninfected or infected with FPV 2 hr previously*

Expt	Reaction mixture	GTP(^{3}H) incorporated (counts per min per mg of protein)[a]	
		Uninfected	Infected
A	Complete	409	596
	Omit ATP, CTP, UTP	0	0
	Omit Mg^{++}	406	579
	Omit Mn^{++}	118	139
	Omit Mg^{++}, Mn^{++}	41	61
	Add ribonuclease, 10 μg	0	0
B	Complete	754	998
	Omit exogenous DNA	624	813
	Omit $(NH_4)_2SO_4$	322	427
	Add deoxyribonuclease 50 μg[b]	0	0

[a] These values divided by 6 correspond to micromicromoles of GTP incorporated into acid-insoluble product per milligram of protein.

[b] Ribonuclease-free.

TABLE 2. *Effect of actinomycin D on DNA-dependent RNA polymerase activity from chick embryo fibroblast cells, uninfected or infected with FPV 2 hr previously*

Actinomycin D in reaction mixture[a] (μg)	Polymerase activity (%)	
	Uninfected	Infected
0	100	100
2	81	61
5	42	34
10	15	15
50	0	0

[a] The complete reaction mixture was as described in Materials and Methods. Actinomycin D was added just prior to the enzyme preparation, at the concentrations indicated.

somal, or cell sap fractions of the infected or control cells.

The results of varying the assay conditions are shown in Table 1. It is apparent that polymerase activity from control or infected cells is dependent upon the presence of DNA template, high ionic strength due to ammonium sulfate, four nucleoside triphosphates, and Mn^{++}. However, Mg^{++} can partially replace Mn^{++}, and is required for maximal activity.

No radioactively labeled acid-insoluble material was recovered when ribonuclease (10 μg) was present in the reaction mixture. Furthermore, when the product of the reaction, following the third perchloric acid precipitation, was treated with 20 μg of ribonuclease, as described by Baltimore and Franklin (3), no acid-insoluble material was recovered. This suggests that the product of the reaction is RNA.

Although the reaction was totally dependent on the presence of DNA, as shown by the inhibitory effect of deoxyribonuclease, omission of exogenous DNA reduced enzyme activity by only 20%. Presumably, the remaining activity was associated with the large amounts of DNA present in the enzyme preparation.

Actinomycin D added at various concentrations to the reaction mixture inhibited the activity of enzyme prepared from infected or control cells to a similar extent (Table 2).

Changes in polymerase activity during infection. Changes in polymerase activity were studied at various times up to 6 hr postinfection. In the nuclei of virus-infected cells, DNA-dependent RNA polymerase activity decreased slightly in the first hour postinfection, and then increased sharply to about 60% above control cell activity at 2 hr. Thereafter, the activity in the virus-infected cells decreased to reach control level by 4 hr and to 50% below control level by 6 hr postinfection (Fig. 5). The increase in polymerase activity in the infected cells at 2 hr was a constant finding and is statistically significant ($P < 0.01$).

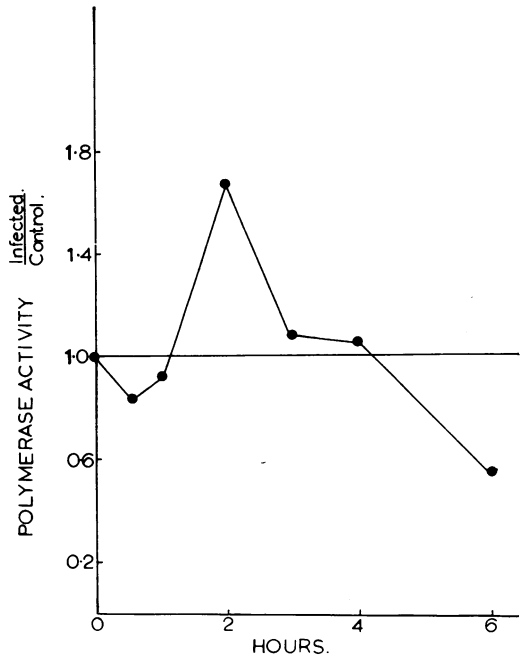

FIG. 5. *DNA-dependent RNA polymerase activity of nuclear suspensions prepared from chick embryo fibroblast cells at various times after FPV infection. Results are expressed as the ratio of polymerase activity (counts per min per mg of protein) of infected to that of uninfected cells at each time. Points represent a mean value of from three to six experiments.*

RNA-dependent RNA polymerase. RNA-dependent RNA polymerase activity was assayed according to the method described by Martin and Sonnabend (19). With the above system, in which assays are made in the presence of actinomycin D, it has not been possible to demonstrate any significant level of RNA-dependent RNA polymerase activity in either the nuclei or microsomes of FPV-infected cultures of chick embryo fibroblasts.

DISCUSSION

The initial depression in cellular DNA-dependent RNA-polymerase activity which we observed during the first hour after infection with FPV is similar to that found during infection of cells with mengovirus or poliovirus (2, 14). However, in the latter cases, cellular RNA polymerase activity continued to be depressed during the period of virus growth, whereas with FPV there was a rise in DNA-dependent RNA polymerase activity between 1 and 3 hr after infection. That

this rise was not due to a new RNA-dependent polymerase is suggested by the dependence of the observed activity on DNA template, ammonium, sulfate, and Mn^{++} ions, and its inhibition by actinomycin D, in contrast to the new virus-induced RNA polymerases described so far (18, 20). Thus, we have no information at present to suggest that the increase in enzyme activity which we observed represents anything more than the enhancement of normal cellular enzyme activity, and it could be that this is the result of some non-specific derangement of cellular metabolism in the virus-infected cells. However, the coincidence of the increase in DNA-dependent RNA polymerase activity with the increase in RNA synthesis between 1 and 3 hr postinfection (Fig. 2) suggests that this enzyme may be concerned in some way with the formation of FPV RNA. If so, it might be possible to detect differences between the uninfected and infected cell polymerases as to either template requirement or the nature of the in vitro reaction product. Experiments along these lines are complicated by the fact that the nucleic acid of mature influenza virus occurs in several pieces, similar in size to the host cellular 18*S* ribosomal RNA (6, 7, 21).

The decrease in polymerase activity during the period of mature virus formation from 3 hr post-infection also coincides with a decrease in RNA synthesis of infected relative to control cells. This decrease probably reflects the destructive effect of virus replication on cellular RNA synthesis, and may well be superimposed on a pattern of continued synthesis of viral RNA, as suggested by Scholtissek et al. (23).

Whereas we could detect no significant RNA-dependent RNA polymerase activity in cells infected with FPV, two groups have reported such an enzyme to be present in other influenza-infected tissues (9, 13). Glasky and Holper (9) claimed to have shown an RNA-primed RNA polymerase in the microsomal fraction of calf kidney cells 12 hr after infection with an influenza B virus. However, this enzyme was inhibited by actinomycin D in vitro, and was present in comparatively high (20%) concentration in uninfected cells, in contrast to other RNA-primed RNA polymerases so far described (18). Ho and Walters (13) described an RNA polymerase in a chorioallantoic membrane supension infected with an influenza A (PR8) virus. The activity appeared within 1 hr after infection and reached a peak at 3 hr, but it required Mg^{++} rather than Mn^{++}, it was not inhibited by actinomycin D in vitro, and it appeared in the microsomal fraction of the cell, in contrast to the DNA-dependent enzyme we have described. Since it is well known that influenza virus RNA is formed in the nucleus of its host cells (5, 16, 25), this increase in the activity of a cytoplasmic RNA polymerase, as shown by Ho and Walters (13), may not necessarily be directly related to virus RNA replication.

There are only three known types of RNA viruses (reovirus, the avian tumor viruses, and influenza virus) whose replication is inhibited by actinomycin D. In no case is the basis of the inhibition clearly understood, but at least for reovirus it may be due to secondary effects of actinomycin, since inhibition is dependent upon comparatively high concentrations of the drug, and the length of time for which the cells are pretreated (8, 26). On the other hand, actinomycin inhibition of the avian tumor viruses (28, 29) appears to be related to a requirement for the synthesis of new DNA which acts as a primer for virus RNA replication (1). Attempts to detect a new RNA-dependent RNA polymerase in cells infected with these viruses have so far proved unsuccessful (30).

If, as our results suggest, cellular DNA-dependent RNA polymerase is involved in the replication of influenza virus, this would explain why virus RNA synthesis is confined to the nucleus of infected cells and why the growth of influenza virus is inhibited by actinomycin D.

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