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RNA-Dependent RNA Polymerase in Nuclei of Cells Infected with Influenza Virus

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Nuclei purified from chicken embryo fibroblast cells infected with influenza (fowl plague) virus contain an RNA-dependent RNA polymerase. The in vitro activity of this enzyme is insensitive to actinomycin D, and is completely destroyed by preincubation with ribonuclease. Enzyme induction is prevented if cells are treated with actinomycin D or cycloheximide at the time of infection. RNA-dependent RNA polymerase activity increases rapidly in cell nuclei from 1 h postinfection, reaches a maximum at 3 to 4 h, then declines; a similar RNA polymerase activity in the microsomal cell fraction increases from 2 h postinfection and reaches a maximum at 5 to 6 h. The characteristics of the nuclear and microsomal enzymes in vitro are similar with respect to pH and divalent cation requirements. The in vitro products of enzyme activity present in the nuclear and microsomal fractions of cells infected for 3 and 5 h were characterized by sucrose density gradient analysis, and annealing to virion RNA. The microsomal RNA polymerase product contained 67 and 93% RNA complementary to virion RNA at 3 and 5 h, respectively; for the nuclear RNA polymerase product these values were 40% in each case.

A number of RNA polymerases associated with influenza virus replication have been described. The influenza virion contains an RNA-dependent RNA polymerase (transcriptase) which synthesizes in vitro RNA complementary to viral genome RNA (3, 24, 30); the function of this enzyme in the infected cell has not yet been elucidated. Early in the replicative cycle, there is a stimulation in DNA-dependent RNA polymerase activity of host cell nuclei (6, 22), which may be related to the unusual sensitivity of influenza virus growth to inhibitors of DNA function (1). Finally, RNA-dependent RNA polymerases have been detected in both the microsomal and the nuclear fractions of infected cells (14, 19–21, 26, 29, 32). The in vitro product of the microsomal enzyme has been characterized (21, 27); it contains virus-specific RNA, much of which is complementary to RNA from influenza virions. The nuclear RNA-dependent RNA polymerase is of low specific activity, and little is known of its properties (4); in particular, it is not clear whether the observed enzyme activity in the nucleus merely represents contamination of this cell fraction by microsomes.

In this communication we describe the appearance and properties of RNA-dependent RNA polymerase activity in nuclei of cells infected with influenza virus, and compare the characteristics of this enzyme with those of the enzyme present in the microsomal fraction.

MATERIALS AND METHODS

Chemicals and buffers. Actinomycin D was a gift from Merck, Sharp & Dohme Ltd., Hoddesdon, Herts. α-Amanitin was purchased from Boehringer Ingelheim Ltd., Isleworth, Middlesex. Creatine phosphate, creatine phosphokinase, ATP, CTP, GTP, UTP, reduced diphosphopyridine nucleotide (NADH), and cytochrome *c* were purchased from Boehringer and Sohne, Mannheim. Cycloheximide, 2-mercaptoethanol, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co., London. Ribonuclease A and deoxyribonuclease 1 (electrophoretically purified free of ribonuclease) were obtained from Worthington Biochemical Corp., Freehold, N.J.; ribonuclease T_1 was obtained from Miles-Seravac Co. Ltd., Maidenhead, Berks.

Labeled nucleoside triphosphates, obtained from The Radiochemical Centre, Amersham, Bucks., were ^{3}H-GTP, 10 Ci/mmol, and ^{3}H-UTP, 3.6 or 10 Ci/

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mmol. SSC buffer was 0.15 M NaCl; 0.015 M Na citrate; pH 7.0. NTE buffer was 10 mM Tris-hydrochloride; 100 mM NaCl; 1 mM EDTA; pH 7.4.

Cells and virus. Monolayer cultures of chicken embryo fibroblast cells were prepared as previously described (6) and grown in medium 199 containing 10% calf serum on 15-cm glass petri dishes maintained at 37 C. For infection, cell sheets were washed in phosphate-buffered saline (PBS), then exposed to allantoic fluid containing influenza virus (fowl plague, Rostock strain, FPV) diluted so as to provide an input multiplicity of infection of approximately 20 plaque-forming units per cell. After adsorption at 37 C for 30 min the virus suspension was discarded and replaced by 30 ml of maintenance medium (199 containing 2% calf serum). Zero time in all experiments corresponded to the time of addition of maintenance medium.

Cell fractionation. Cells were homogenized and the microsomal fraction was prepared as described previously (21). The crude nuclear fraction was further purified as follows. The nuclei were washed in 0.32 M sucrose, 1 mM $MgCl_2$, then resuspended in a further 1.2 ml of this solution in a 5-ml centrifuge tube. A 3.8-ml amount of 2.4 M sucrose, 1 mM $MgCl_2$ was then slowly added to the nuclear preparation by using a 5-ml syringe attached to a 19-gauge needle. The suspension was mixed thoroughly by using the syringe, and finally centrifuged at 80,000 $\times$ *g* for 30 min. Nuclei were recovered as a white pellet whilst unbroken cells and cytoplasmic debris formed a yellow band at the top of the tube. The nuclear pellet was resuspended in 0.32 M sucrose, 1 mM $MgCl_2$ by using a loose-fitting Dounce homogenizer, then Nonidet P-40 (NP-40) was added to a final concentration of 1% and the suspension was thoroughly mixed at room temperature, during 1 min. The pure nuclei were finally sedimented at 1,000 $\times$ *g* for 5 min, and washed twice more in 0.32 M sucrose, 1 mM $MgCl_2$ before use.

NADH cytochrome c reductase assays. Enzyme reactions were carried out in a total volume of 3.5 ml in a spectrophotometer cuvette. A 3-ml amount of phosphate buffer, pH 7.6, was mixed with 0.1 ml of cytochrome *c* (100 nmol) and 0.3 ml of cell fractions (approximately 500 μg of protein in 0.32 M sucrose, 1 mM $MgCl_2$), and the reaction was started by adding 0.1 ml NADH (100 nmol). Enzyme activity was determined by measuring the increase in optical density at 555 nm using a Pye-Unicam SP 500 recording spectrophotometer, and the results expressed as ΔE_{555} per minute per milligram of protein.

RNA-dependent RNA polymerase assay. The standard reaction mixture for polymerase assay contained, per 0.2 ml; Tris-hydrochloride (pH 8.5), 50 μmol; $MgCl_2$, 2 μmol; 2-mercaptoethanol, 3 μmol; creatine phosphate, 3 mg; creatine phosphokinase, 30 μg; actinomycin D, 20 μg, ATP and CTP, 0.036 μmol each; and either UTP, 0.036 μmol with ^{3}H-GTP, 5 nmol, or GTP, 0.036 μmol with ^{3}H-UTP, 5 nmol. Cell fractions were added in a volume of 0.1 ml of 0.32 M sucrose in 1 mM $MgCl_2$ to the reaction mixtures, which were incubated at 28 C for 20 min, unless otherwise stated, before determination of acid-insoluble radioactivity in duplicate samples (21). In all experiments, nuclei or microsomes from uninfected cells were assayed as controls, and the observed incorporation was subtracted from the total acid-insoluble radioactivity incorporated by infected cell fractions.

DNA-dependent RNA polymerase assay. This was carried out using the reaction mixture for RNA polymerase II as previously described (22).

Protein determination. Protein was determined by the method of Lowry et al. (17) with bovine serum albumin as standard.

Extraction of RNA from RNA polymerase reactions. (i) Microsomal RNA-dependent RNA polymerase product. A 3-ml amount of reaction mixture was added to 1 ml of the microsome suspension. After a 20-min reaction, sodium EDTA (10 mM) and SDS (1%) were added. This suspension was mixed vigorously for 5 min at room temperature. An equal volume of ice-cold NTE-saturated phenol was then added and the mixture was shaken at 4 C for 45 min. The phases were separated by centrifugation at 2,000 $\times$ *g* for 10 min and the upper aqueous phase was reextracted with an equal volume of NTE-saturated phenol at 4 C for 15 min. This process was repeated until no interface remained between the two phases. The RNA in the final aqueous phase was precipitated with ethanol (2 volumes) and 5 M sodium acetate (0.1 volume) at -20 C for a minimum of 1 h. The RNA was removed from the suspension by centrifugation at 3,000 $\times$ *g* for 15 min, air dried, dissolved in 1 ml NTE buffer, and reprecipitated with ethanol and acetate as above. This reprecipitation procedure was repeated once more before the RNA was used for further analysis.

(ii) Nuclear RNA-dependent RNA polymerase product. The RNA was extracted from nuclei by the hot phenol-chloroform procedure described by Penman (25). A 3-ml amount of reaction mixture was added to 1 ml of a suspension of nuclei. After a 20-min reaction, sodium EDTA and SDS were added as for microsomes (see above). This suspension was mixed vigorously for 5 min at room temperature. One volume of warm NTE-saturated phenol was then added and, after mixing, the contents of the tube were heated to 55 C for 1 min followed by the addition of 1 volume of chloroform, mixing and heating again to 55 C. The phases were then separated by centrifugation at 2,000 $\times$ *g* for 10 min; the lower phenol-chloroform phase was removed, leaving the aqueous phase and interface which were reextracted three times at 55 C with 2 volumes of chloroform. The final aqueous phase was precipitated with ethanol (2 volumes) and 5 M sodium acetate (0.1 volume) at -70 C overnight. The RNA-DNA precipitates were recovered from the suspension by centrifugation at 3,000 $\times$ *g* for 15 min and inverted over tissue paper for drying. The DNA was removed by incubation in 2 ml of nuclease buffer containing DNase (100 μg/ml) for 30 min at 37 C. This suspension was reextracted with phenol at 4 C for 30 min and the final aqueous phase obtained after centrifugation was precipitated with ethanol and 5 M Na acetate. The RNA was used for further analysis after two reprecipitations.

Sucrose-gradient analysis of RNA. Linear 5 to

20% gradients of sucrose in NTE buffer were prepared and fractionated as previously described (13).

Purification of FPV. Infected allantoic fluid was mixed with 1 volume of ammonium sulfate, saturated solution at 4 C, then centrifuged for 15 min at 10,000 rpm in 250-ml samples. The pellet was resuspended in 0.1 volume PBS with a Teflon-glass homogenizer, the centrifuged in an SW27 rotor at 25,000 rpm for 3 h in a discontinuous gradient containing in the upper phase, 25% sucrose, 25% glycerol, 25% potassium tartrate (SGT) adjusted to 1.197 density by diluting 2 parts to 1 part water; and in the lower phase SGT was diluted 7 parts to 1 part water to achieve density 1.295. The sucrose mixture was removed from the virus band after centrifugation by passage through a column of coarse-grade Sephadex G50, then recentrifuged (60 min at 16,000 rpm) through a rate-zonal gradient prepared from SGT 1 part, water 7 parts (density 1.027), and equal parts of SGT and water (density 1.148). After collection, the virus band was concentrated on an SGT cushion by centrifugation for 90 min at 26,000 rpm, and equilibrated into PBS through a Sephadex G50 column before use.

Extraction of RNA from purified FPV. RNA was extracted from purified virus as described for microsomal RNA-dependent RNA polymerase product.

RNA-RNA annealing. Samples of RNA for annealing assay were mixed in a total volume of 50 μliters of 2 $\times$ SSC buffer in 1-ml sealed glass ampoules. After incubation at 70 C for 1 h, the mixtures were slowly cooled overnight to room temperature. The contents of each ampoule were made up to 0.5 ml with 2 $\times$ SSC and transferred to a conical centrifuge tube. A 0.5-ml amount of 2 $\times$ SSC containing 50 μg of ribonuclease A and 25 U of ribonuclease T_1 were added, and after incubation at 28 C for 30 min, acid-insoluble radioactivity was determined (21). In specified cases the product RNA species were heat-denatured before annealing. The RNA sample, in 2 $\times$ SSC, was heated to 115 C for 1 min, then rapidly cooled by immersion in a solid CO_2-ethanol freezing mixture.

Hemagglutinin assay. This was carried out as previously described (6).

RESULTS

Presence of RNA-dependent RNA polymerase activity in nuclei. It has been shown previously (19, 29, 32) that the specific activity of RNA-dependent RNA polymerase in crude nuclei constitutes 10 to 15% of the microsomal activity in cells infected with influenza virus for 6 h. To determine whether the enzyme activity of the nuclear fraction was due to cytoplasmic contamination, three methods for purifying cell nuclei were investigated. In the first method, crude nuclei were centrifuged through 1.6 M sucrose over a cushion of 2.2 M sucrose as described by Blobel and Potter (5). This technique proved unsuitable for chicken embryo fibroblast cells, as nuclei became trapped with whole cells at the interface. The second method was that described by Penman (25), in which crude nuclei were treated with a mixture of two detergents, Tween 80 and sodium deoxycholate causing removal of the outer nuclear membrane and associated cytoplasmic tags. This treatment destroyed nuclear RNA-dependent RNA polymerase activity completely (Table 1), but as microsomal RNA polymerase activity was also destroyed, no conclusions could be drawn as to the existence of the enzyme within the nuclear fraction. The sensitivity of influenza virus-induced RNA polymerase activity to deoxycholate has been noted by others (10).

The third method, a modification of the Chauveau technique (9, 15) with an additional non-ionic detergent treatment as described in Materials and Methods, proved the most suitable for purification of chicken embryo fibroblast nuclei. Preparations of nuclei purified by this procedure were routinely checked by phase contrast microscopy and found to be completely free from visible cytoplasmic contamination. Examination of nuclei by phase-contrast microscopy, although a useful guide to the amount of whole cell contamination, permits no valid quantitative conclusions on the level of cytoplasmic contamination. For this reason it has become common practice to estimate the level of nuclear purity by assaying for the presence of enzymes known to exist in cytoplasm alone (8). The enzymes NADH-linked cytochrome *c* reductase and α-amanitin-sensitive DNA-dependent RNA polymerase were therefore assayed on cell fractions during purification as cytoplasmic and nuclear markers, respectively. Crude nuclei possessed measurable cytochrome *c* reductase activity, but this was lost after NP-40 treatment; by contrast, DNA-dependent RNA polymerase activity progressively increased during purification of the nuclei (Table 1).

It has been reported that impure nuclei from influenza virus-infected cells will agglutinate chick erythrocytes due to contamination with cytoplasmic hemagglutinin (HA) (11). This was confirmed, as shown in Table 1. The HA activity of nuclei at various stages of purification was standardized in relation to the number of nuclei present and expressed as HA activity per 10^6 nuclei. There was a progressive decrease in specific HA activity as washed nuclei were purified through sucrose and then detergent treated, the latter procedure reducing activity completely. The loss in HA caused by NP40 treatment was due to loss of cytoplasm and not to a destructive effect of the detergent on HA activity since vigorous mixing for 1 min with 1%

TABLE 1. *Enzyme activities in fractions from CEF cells infected with influenza virus*

Fraction[a]	NADH cytochrome c reductase (E_{555} per min per mg of protein)	Hemagglutinin titer (U/10^6 nuclei)	DNA-dependent RNA[b] polymerase act (^{3}H-UMP incorporated, counts per min per mg of protein)	RNA-dependent RNA polymerase act. (^{3}H-UMP incorporated, counts per min per mg of protein)
Homogenized cells	0.106	ND[c]	4,193	6,869
Washed nuclei	0.083	38	14,225	1,600
Nuclei sedimented through 1.9 M sucrose	0.056	12	20,440	2,116
Nuclei sedimented through 1.9 M sucrose, treated with 1% NP-40	Not detected	Not detected	30,583	4,417
Microsomes	0.116	ND	1,942	102,142
Nuclei treated with Tween-deoxycholate	ND	ND	ND	0
Microsomes treated with Tween-deoxycholate	ND	ND	ND	0

[a] Cells were fractionated 5 h after infection with FPV as described in Materials and Methods.
[b] DNA-dependent RNA polymerase II was assayed in presence of Mn^{2+} and high salt as described elsewhere (22).
[c] ND, not done.

NP40 had no effect on the HA titer of purified virus.

Cells infected for 5 h with FPV were assayed for RNA-dependent RNA polymerase activity at various stages of nuclear purification. In contrast to the results obtained for cytochrome c reductase and HA activities, progressive purification of the infected nuclei led to an increase in RNA-dependent RNA polymerase activity. Calculation of the total activity in cell homogenates, microsomes, and nuclei (allowing for 33% recovery of nuclei after the sucrose step) showed that at 5 h postinfection, microsomes contained 67% of the total RNA-dependent RNA polymerase of the infected cell, and nuclei 9%. All subsequent experiments on the properties of the nuclear enzyme and its RNA product were performed on nuclei purified by centrifugation through 1.9 M sucrose, treated with NP40, and finally washed twice in 0.32 M sucrose, 1 mM $MgCl_2$.

Properties of the nuclear RNA-dependent RNA polymerase in vitro. The RNA-dependent RNA polymerase activity in the microsomal fraction of cells infected with influenza virus was previously shown to be dependent on the addition of all four ribonucleoside triphosphates, and to be sensitive to ribonuclease, but insensitive to deoxyribonuclease added during the reaction (21, 26, 29, 32). Table 2 shows the requirements for the RNA-dependent RNA polymerase activity of nuclei from cells infected for 5 h with FPV. Omission of any one of the three unlabeled ribonucleoside triphosphates from the reaction caused a decrease in enzyme activity. Omission of all three ribonucleoside triphosphates from the reaction abolished RNA polymerase activity completely. Pancreatic ribonuclease (50 μg/ml), when added at the beginning of the reaction, completely inhibited the incorporation of ^{3}H-UTP into acid-insoluble material, but when added at the end of a 20-min reaction, inhibited incorporation by 70%. The 30% activity insensitive to ribonuclease is presumably due to the proportion of double-stranded RNA in the reaction product (see below).

TABLE 2. *In vitro requirements of the nuclear RNA-dependent RNA polymerase*

Reaction mixture	RNA-dependent RNA polymerase act[a]
Complete	11,010
Minus ATP, UTP, CTP	0
Minus ATP	4,140
Minus UTP	2,290
Minus CTP	8,280
Plus ribonuclease before the reaction[b]	0
Plus ribonuclease at the end of the reaction	2,650

[a] RNA polymerase activity is expressed as ^{3}H-GMP (counts/min) incorporated into acid-insoluble material per milligram of protein in a 20-min assay.
[b] Nuclei were incubated with 50 μg of pancreatic ribonuclease per ml for 20 min at 28 C either before or at the end of the reaction. Nuclei were purified from cells infected for 5 h with FPV.

Actinomycin D (100 μg/ml) was routinely included in the RNA polymerase reaction mixture; the results of other experiments (not shown) demonstrated that no reduction in enzyme activity occurred when either deoxyribonuclease (up to 100 μg/ml) or α-amanitin (25 μg/ml) were incubated with nuclei for 20 min before the reaction. Thus the enzyme in the nucleus of cells infected with FPV is an RNA-dependent RNA polymerase. In the following experiments, the properties of this enzyme were compared with those of the RNA-dependent RNA polymerase present in infected cell microsomes.

Divalent cation and pH requirements. Microsomal and nuclear fractions from cells infected for 5 h with FPV were assayed for RNA-dependent RNA polymerase activity in the presence of varying concentrations of $MgCl_2$ or $MnCl_2$. In a 20-min reaction Mg^{2+} was much more effective than Mn^{2+} in stimulating enzyme activity of both cell fractions. For future experiments $MgCl_2$ was used at a final concentration of 6.6 mM for the assay of both the nuclear and microsomal enzymes.

The effect of varying pH on RNA-dependent RNA polymerase activity was studied by using nuclei and microsomes from cells infected for 5 h with FPV. There was an optimum for both fractions at about pH 8.5; this pH was therefore used in all further experiments.

Protein concentration in the reaction mixture. The RNA polymerase activity of reaction mixtures containing increasing concentrations of microsomal and nuclear protein was determined. In both cases incorporation of label into acid-insoluble counts was proportional to the amount of protein present up to a critical level beyond which addition of further protein became rate limiting. The microsomal enzyme activity was proportional to the amount of protein present at concentrations below 175 μg/0.3 ml of assay, the nuclear enzyme activity at concentrations below 250 μg/0.3 ml of assay. In polymerase assays the concentrations of microsomal and nuclear protein used were always well below these critical levels (usually about 100 μg/0.3 ml of assay).

Time course of appearance of enzyme activity in nuclei and microsomes. Scholtissek and Rott (29) reported previously that in both microsomes and impure nuclei of CEF cells infected with FPV, polymerase activity continued to increase up to at least 9 h after infection. Figure 1 shows the results of an experiment in which the cells from groups of five infected monolayer cultures were harvested at hourly intervals up to 7 h postinfection. The microsomes and nuclei of these cells were assayed for RNA-dependent RNA polymerase and virus-induced activity was estimated by subtraction of uninfected cell control values. In contrast to the result obtained by Scholtissek and Rott, nuclear activity appeared during the first hour postinfection, reached a maximum at 3 h after infection and thereafter declined with time. This result was consistently obtained in several separate experiments. The enzyme in the microsomes was first detected between 1 and 2 h postinfection, increased up to 5 to 6 h postinfection, and had decreased by 7 h. Previous analyses of the microsomal enzyme reaction and its product RNA employed the fraction of cells infected for 5 to 6 h (21). Further characterization of the in vitro reaction kinetics and product RNA species used as enzyme source the microsomal and nuclear fractions from cells infected for both 3 and 5 h.

In vitro kinetics of the RNA-dependent RNA polymerase reactions. Nuclei or microsomes from cells infected for 3 or 5 h were added in a volume of 1 ml to a 6-ml reaction

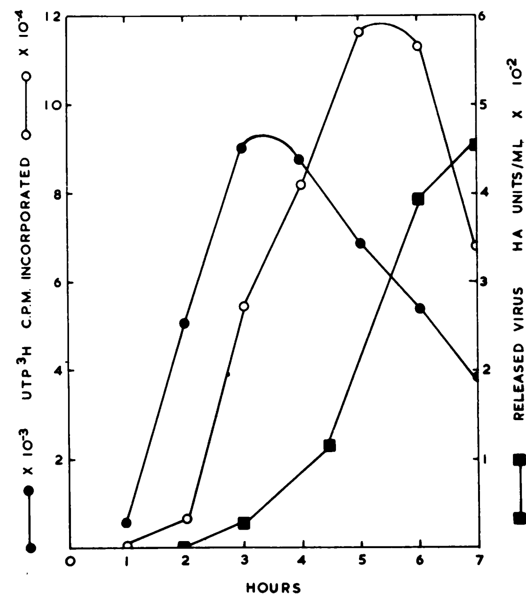

FIG. 1. *Time course of appearance of RNA-dependent RNA polymerase activity in CEF cells infected with influenza virus. CEF cells were infected with FPV. At hourly intervals the medium was assayed for released virus, and cells (from five 15-cm dishes) were harvested and fractionated at 0 C, then microsomes and nuclei assayed for RNA-dependent RNA polymerase activity for 20 min at 28 C, as described in Materials and Methods. Symbols:* ●, *nuclei;* ○, *microsomes;* ■, *viral HA titer.*

mixture. At intervals sets of 4 × 0.2-ml samples were taken from the reaction and immediately frozen in solid carbon dioxide-ethanol mixture. Two of the samples were assayed for total acid-insoluble radioactivity, two were treated with pancreatic ribonuclease (50 μg/ml) for 30 min at 37 C, and subsequently were assayed for acid-insoluble radioactivity. The kinetics of incorporation of label by the microsomal enzyme at 3 or 5 h were essentially the same; this was true also for the nuclear enzyme (Fig. 2). There were two obvious differences between the microsomal and nuclear enzyme kinetics: (i) ^{3}H-UTP was incorporated by the microsomal enzyme in a linear fashion for at least 45 min. There was no increase in incorporation by nuclear enzyme beyond 15 to 20 min. (ii) The proportion of ribonuclease-resistant material synthesized by the 5-h microsomal polymerase throughout the 60-min reaction was 50 to 60%. Only 25 to 30% of the RNA synthesized by the nuclear polymerase was ribonuclease-resistant.

The proportion of ribonuclease-resistant material did not alter significantly during the reaction with any of the four fractions studied. Therefore, as shown previously by in vitro pulse-chase experiments (21), there was no obvious product-precursor relationship between double-stranded and single-stranded RNA. The nuclear activity at 3 h (Fig. 2A) was higher in relation to microsomal enzyme activity than at 5 h (Fig. 2B), reflecting the difference in rates of appearance of the enzymes in the two cell fractions.

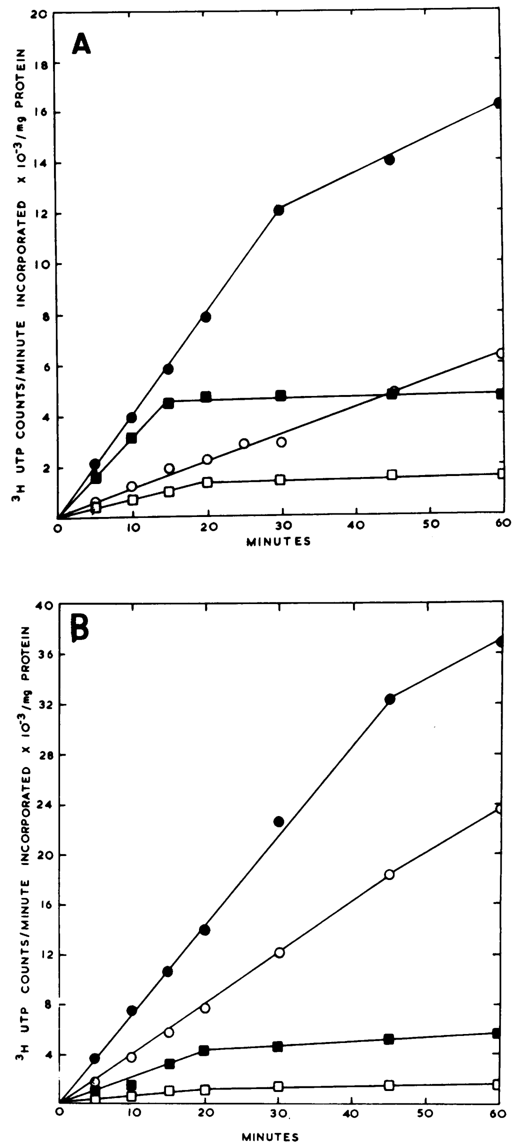

FIG. 2. *Kinetics of incorporation of ^{3}H-UTP into acid-insoluble material. Nuclei or microsomes from CEF cells infected for 3 h (A) or 5 h (B) with FPV were added in a volume of 1 ml to a 6-ml reaction mixture. At the time intervals indicated 4 × 0.2-ml samples were removed from each reaction mixture, and frozen immediately in a solid CO_2-ethanol mixture. Two of the samples were assayed for acid-insoluble radioactivity directly, and two were first incubated with pancreatic ribonuclease (50 μg/ml) for 20 min at 28 C before determining acid-insoluble radioactivity. Symbols: ●, microsomes, total RNA; ○, microsomes, ribonuclease-resistant RNA; ■, nuclei, total RNA; □, nuclei, ribonuclease-resistant RNA.*

Effect of inhibitors on the synthesis of RNA-dependent RNA polymerase. Although it is likely that the appearance of new RNA-dependent RNA polymerase activity in the cell requires protein synthesis, this is not necessarily the case since the virus may be inducing a change in template specificity of a host DNA-dependent RNA polymerase independent of protein synthesis. However, as shown in Table 3 protein synthesis was found to be necessary for the synthesis of both microsomal and nuclear enzymes. Cycloheximide, added at the time of virus infection, completely inhibited the synthesis of the enzyme in both cell fractions (Table 3). In the same experiment actinomycin D was added to the cells at the time of infection to separate cultures. This drug, an inhibitor of cell RNA synthesis, also prevented the synthesis of the RNA-dependent RNA polymerase.

RNA products of the in vitro polymerase reactions. (i) Sucrose density gradient sedimentation analysis. Nuclei and microsomes extracted from uninfected, 3-h FPV-infected

and 5-h FPV-infected cells (13 monolayer cultures for each) were used in a 20-min RNA polymerase reaction. The RNA product from the reaction using microsomes as the enzyme source was extracted by using the cold phenol-SDS procedure described previously (21). When this procedure was applied to the nuclear RNA polymerase products the RNA extracted was of low molecular weight; for this reason, the hot phenol-chloroform procedure described by Penman was used to extract nuclear RNA polymerase products. This method reproducibly extracted 45*S* ribosomal precursor RNA from whole chicken embryo fibroblast cells (13).

Five to 20% sucrose density gradients (16 ml) containing 0.5% SDS, were used for sedimentation analysis of the RNA-dependent RNA polymerase products; the gradients were run at 65,000 × *g* for 16 h and were fractionated automatically by using an ISCO fractionator fitted to a UV recording spectrophotometer set to record at 260 nm. Figure 3 shows the profiles of total and ribonuclease-resistant microsomal products (a)-(c) from 3-h FPV-infected, 5-h FPV-infected and uninfected cells, respectively. An equal proportion (of that originally extracted) of each of these species of RNA was applied to gradients to allow for direct comparison. Equal samples were used in analysis of untreated and ribonuclease-treated products. The profiles of products synthesized by microsomes from 3- or 5-h FPV-infected cells were essentially the same, consisting of a major species at 18*S* and smaller amounts of a range of smaller species down to 8*S*. Each of the microsomal products had its own endogenous optical density profile (260 nm) due to the unlabeled ribosomal RNA in the preparation. This allowed for exact determination of size. Although previous analyses of microsomal products have demonstrated some material between 14 and 18*S*, this was usually inferior in amount to smaller material sedimenting at 8*S* or less than 4*S* (21, 26, 33). The ribonuclease-resistant material, which formed a high proportion of both the 3- and 5-h microsomal products, sedimented as a homogeneous band in the 11*S* region of the gradient, as previously reported (21). There was a great deal more label in the 11*S* region of the gradient of ribonuclease-treated products than in the corresponding gradients of untreated products. In nontreated samples, therefore, it must be assumed that the double-stranded RNA sediments together with the bulk of the enzyme reaction product, at 18*S*. This suggests that the double-stranded material is complexed to a large amount of single-stranded RNA in the form of 'replicative intermediate'.

Figure 3 (d)-(f) shows the profiles of 3-h FPV-infected, 5-h FPV-infected, and uninfected nuclear RNA polymerase products. The results for FPV-infected products were obtained by subtraction of the uninfected profile which constituted a significant proportion of the total RNA extracted (26%). In both cases, the FPV-infected nuclear RNA polymerase products sedimented heterogeneously, with a major RNA species at 14*S*, and shoulders on either side of this peak. The ribonuclease-resistant material constituted a smaller proportion of the total RNA than was the case for the microsomal enzyme products, and sedimented as a broad band at 12 to 14*S*. Cold phenol extraction of microsomal products, or hot-phenol extraction of nuclear products, appeared to have no effect on the proportion of the RNA which was ribonuclease-resistant since, after extraction, this proportion was unchanged from that observed in the kinetic experiments (Fig. 2). The small amount of radioactivity in the uninfected microsomal RNA polymerase product sedimented throughout the gradient but mainly at 18 and 28*S*, coinciding with the optical density profile. The uninfected nuclear RNA polymerase product sedimented at 4*S*. Both the uninfected nuclear and microsomal RNA polymerase products were completely sensitive to ribonuclease.

Hybridization of the RNA-dependent RNA polymerase products to influenza virion RNA. It is important in considering the relevance of these RNA polymerases to the virus

TABLE 3. *Effects of inhibitors in vivo on the appearance of RNA polymerase activity in microsomes and nuclei of cells infected with FPV*

Drug	RNA-dependent RNA polymerase act[a]	
	Microsomes	Nuclei
No drug	43,209	4,660
Cycloheximide[b]	120	0
Actinomycin D[c]	504	0

[a] Polymerase activity was assayed on cell fractions prepared 6 h after infection, and is expressed as ^{3}H-UMP (counts/min) incorporated into acid-insoluble material per milligram of protein in a 30-min assay.

[b] Twenty-five micrograms of cycloheximide per ml was added in the medium to cells at the time of virus infection.

[c] One microgram of actinomycin D per ml was added in the medium to cells at the time of infection.

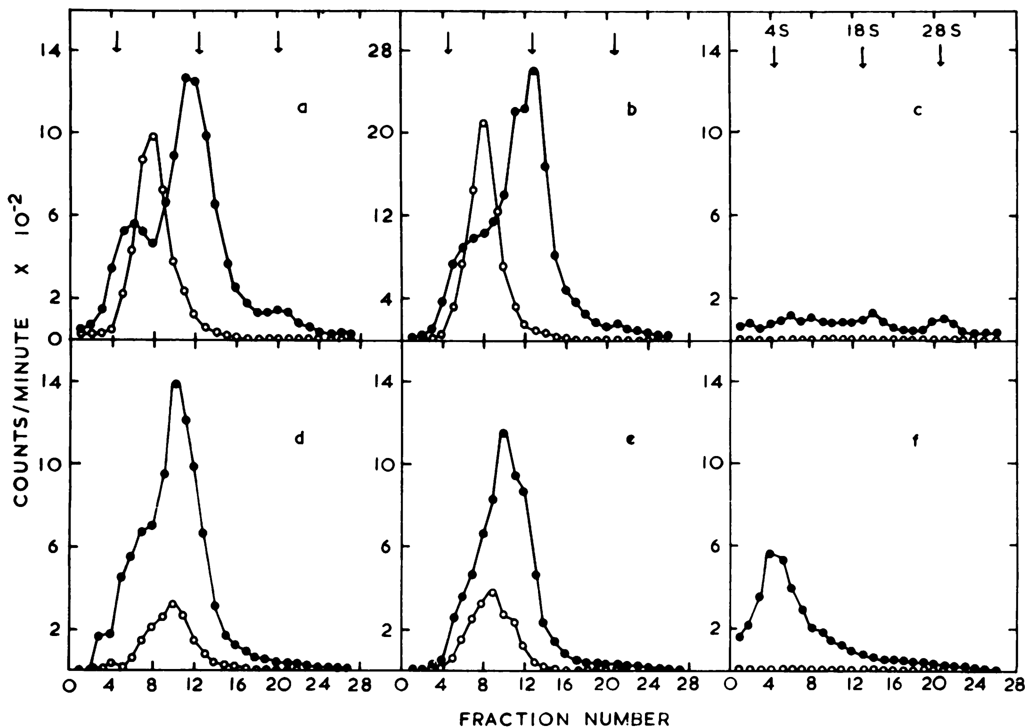

FIG. 3. *Characterization of the in vitro products of the microsomal and nuclear RNA-dependent RNA polymerase reactions. Polymerase product RNA labeled with ^{3}H-UTP was extracted as described in Materials and Methods from 20 min reactions using as enzyme source microsomes and nuclei from cells infected for 3 or 5 h and from uninfected cells. Samples of a 1-ml product RNA in NTE buffer, either untreated (●) or treated with 50 μg of pancreatic ribonuclease per ml for 30 min at 28 C (○) were layered over 16-ml, 5 to 20% sucrose gradients in NTE and centrifuged in Beckman SW27 rotor at 65,000 × g for 16 h. Fractions of 0.63 ml were collected by using an ISCO automatic fractionator and monitored for absorbance at 260 nm by using a recording spectrophotometer. Arrows indicate the positions of 4, 18, and 28S cellular RNA species. (a, b, c) Three-hour, 5-h virus-infected and uninfected microsomal polymerase reaction products, respectively. (d, e, f) Three-hour, 5-h virus-infected and uninfected nuclear polymerase reaction products, respectively.*

replication cycle to characterize the extent of sequence homology or complementarity of the products to viral RNA. A high proportion of the 3- and 5-h microsomal enzyme product was ribonuclease-resistant in the absence of added FPV RNA (vRNA). This was partly due to self-annealing, since the amount of intrinsic ribonuclease-resistance was less in nonannealed samples. A different result was obtained with the nuclear enzyme products, which became slightly less ribonuclease-resistant after self-annealing (Table 4).

On annealing with excess virion RNA the ribonuclease-resistance of the 3- and 5-h microsomal enzyme products increased from 51 to 62% and 67 to 86%, respectively, and that of the 3- and 5-h nuclear enzyme products from 34 to 44% and 38 to 51% (Table 4). This increase in ribonuclease resistance represents the proportion of single-stranded RNA in the product which is complementary to virion RNA (cRNA) but the experiment provides no information about the symmetry of labeling in the product already ribonuclease resistant before the addition of unlabeled virus RNA. To determine this, another set of samples were heat-denatured in order to separate the strands before annealing.

Prior to annealing with unlabeled FPV RNA, the microsomal and nuclear RNA polymerase products were denatured by heating for 1 h at 110 C, followed by rapid quenching in a freezing mixture of solid CO_2-ethanol to prevent re-annealing. This procedure reduced the ribonuclease resistance of all four enzyme products to less than 1% (Table 4) so that all the labeled RNA strands in the mixture were free and, if complementary, able to hybridize to FRV RNA. With saturating amounts of unlabeled viral RNA, the ribonuclease-resistance of the 3- and 5-h microsomal enzyme products increased to

67 and 93%, respectively (Table 4). This was slightly higher than the corresponding levels obtained with nonmelted products. Therefore, all radioactivity in the double-stranded portion of these microsomal polymerase products was in cRNA so that 67% of the 3-h and 93% of the 5-h product was cRNA. In the case of the nuclear polymerase products, there was a slight displacement of label from the duplex after annealing with excess vRNA (Table 4) so that the total ribonuclease-resistant radioactivity was less than with non-melted products. For both 3- and 5-h nuclear RNA polymerase products, the proportion of in vitro synthesized RNA which was complementary was 40%.

DISCUSSION

Nuclei from CEF cells have been purified by a procedure which involves sedimentation through high molarity sucrose followed by mild detergent treatment. These nuclei when examined by phase contrast microscopy were free from visible cytoplasmic contamination, and contained no detectable NADH cytochrome *c* reductase, a cytoplasmic enzyme, or contaminating viral HA when extracted from infected cells. Such nuclei from influenza virus-infected cells contain an RNA-dependent RNA polymerase, the specific activity of which increases as the nuclei are purified. We conclude that the RNA-dependent RNA polymerase occurs within the nuclei, and does not merely reflect cytoplasmic contamination.

Comparison of the properties of the nuclear and microsomal RNA-dependent RNA polymerases revealed no significant differences in requirement for divalent cations or pH optimum. RNA-dependent RNA polymerase activity in purified fowl plague virions is maximal at pH 8.2, and at concentrations of magnesium and manganese of 7 and 1 mM, respectively (Carroll and Mahy, unpublished data); similar results have been reported for the WSN strain of influenza virus (2). The influenza virion and virus-induced cellular RNA polymerase activities cannot be distinguished by these criteria.

One difference between the polymerase activities in microsomes and nuclei is the rate of appearance of the enzyme in the two cell fractions. Polymerase activity reached a maximum in nuclei at 3 h postinfection and then declined, whereas microsomal polymerase activity continued to increase until 5 to 6 h postinfection. As the total activity present, not the rate of synthesis, was measured, it is surprising that RNA polymerase activity in the nucleus should decrease from 3 to 7 h postinfection by 70%. There are perhaps two explanations for this: the enzyme may be unstable, or may migrate into the cytoplasm. No evidence exists regarding the stability of the nuclear enzyme in vivo. However, influenza ribonucleoprotein (RNP) antigen accumulates in the nucleus of infected cells up to 3 h postinfection and is subsequently transported into the cytoplasm (7, 12, 18). The decrease in nuclear RNA-dependent RNA polymerase activity from 3 h may be due to loss of RNP acting as template, or the RNP itself may possess enzyme activity (10).

Reaction catalyzed by nuclei ceased after 20 min compared with 60 min for the microsomal fraction. It has been suggested that failure to re-initiate is a common property of all virus-induced RNA-dependent RNA polymerases as-

TABLE 4. *Hybridization of nuclear and microsomal RNA polymerase products (% ribonuclease resistance of products)*

	3-h Microsomal product[b]	3-h Nuclear product	5-h Microsomal product	5-h Nuclear product
Non-denatured products treatment[a]				
No annealing	43	46	53	40
Annealing in the absence of FPV RNA	51	34	67	35
Annealing in the presence of 80 μg of FPV RNA per ml	62	44	86	52
Denatured products treatment				
No annealing	<1	<1	<1	<1
Annealing in the absence of FPV RNA	45	14	70	14
Annealing in the presence of 80 μg of FPV RNA per ml	67	40	93	40

[a] Non-annealing samples, melted or nonmelted, were kept at 4 C.

[b] Cells were infected with FPV for 3 or 5 h, then nuclear and microsomal fractions were prepared and incubated for 20 min in an RNA-dependent RNA polymerase reaction mixture before extraction of the RNA products as described in Materials and Methods. The RNA products were annealed with saturating concentrations of FPV RNA before determining percentage resistance to ribonuclease.

sayed in crude cell systems (2), so this may apply to both enzymes. The difference in reaction kinetics could be due to limitation in uptake of nucleoside triphosphates into the nuclei in vitro, or to the presence of ribonuclease activity in the nuclear fraction. The latter seems the less likely, since analysis of the total product RNA species on sucrose-density gradients showed RNA similar in size to influenza virion RNA in each case, with no evidence of selective degradation of the nuclear RNA polymerase product.

Hybridization of the 3- and 5-h nuclear and microsomal RNA polymerase products with virion RNA demonstrated that each contained a proportion of cRNA indicating that both enzymes are involved in virus-specific RNA synthesis. The 5-h microsomal polymerase product, as found previously (21, 27), consisted mainly of cRNA. This supports the suggestion that enzyme activity assayed in microsomes 5 h after infection and the virion RNA polymerase may be identical (10).

It has been reported from kinetic analysis of FPV-infected cells that at 3 h postinfection in vivo the rate of virus-specific RNA synthesis is at a maximum, and consists mainly of vRNA (28). In vitro, the 3-h microsomal polymerase product contained 67% cRNA, only slightly less than at 5 h. The nuclear RNA polymerase products contained 40% cRNA in each case (Table 4). The remaining 60% may consist of vRNA, which would implicate the nuclear RNA polymerase activity in virion RNA synthesis in vivo, but as yet we have no direct evidence on this point.

As the viral induced RNA-dependent RNA polymerase activities of both nuclei and microsomes have been shown to be dependent on protein synthesis, it should be possible to relate them to the newly synthesized proteins detected in infected cells. Skehel (31) studied the newly formed polypeptides in CEF cells infected with FPV, and found in addition to the seven structural virus polypeptides, three nonstructural polypeptides of molecular weights 78,000, 23,000, and 11,000. By pulse-chase experiments the largest of these was shown to be a precursor to the two HA polypeptides (31). Therefore, only two small polypeptides are synthesized in the cell apart from the structural polypeptides. Lazarowitz et al. (16) detected a nonstructural protein, with a molecular weight of 25,000, which migrated to the nucleus of the cell and a polypeptide of similar molecular weight has been shown to migrate specifically into the nucleolus of influenza virus-infected cells (33), a site where a nonstructural antigen has been detected in large amounts (11). As this polypeptide, apart from the nucleoprotein, is the only one which migrated into the nucleus it might be related to the nuclear RNA-dependent RNA polymerase activity. The rate of formation of the 23,000 molecular-weight polypeptide (31) is similar to the rate of appearance of nuclear RNA polymerase activity. This polypeptide was not present in RNA polymerase partially purified from the cytoplasm of influenza-infected cells (10), and if it is associated with the RNA-dependent RNA polymerase activity present in the nucleus it is not likely to be a major structural polypeptide of the enzyme.

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