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RNA-dependent RNA Polymerase Activity in Murine Coronavirus-infected Cells

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SUMMARY

The multiplication of murine coronavirus strains A59 or JHM in Sac(−) cells was unaffected by the presence of α-amanitin at concentrations which inhibited the host cell DNA-dependent RNA polymerase activity. In cells infected with the A59 virus strain, actinomycin D-resistant RNA synthesis could readily be detected by pulse-labelling with [^{3}H]uridine; this virus-specific RNA synthesis was not induced in the presence of the protein synthesis inhibitor anisomycin. A new RNA-dependent RNA polymerase activity was detected in the large particle fraction of A59 virus-infected cells. Optimal conditions for enzyme activity *in vitro* were established. Maximum activity occurred 5 h after infection, coincident with the peak of virus-specific RNA synthesis detected by pulse-labelling *in vivo*.

INTRODUCTION

The genome of mouse hepatitis virus (MHV), a coronavirus (Tyrrell *et al.*, 1978), is a single-stranded RNA molecule of 6 × 10^6 to 7 × 10^6 mol. wt. which is polyadenylated at the 3′-terminus, contains a cap structure at the 5′-terminus, and is infectious (Lai & Stohlman, 1978, 1981; Lai *et al.*, 1982; Leibowitz & Weiss, 1981; Wege *et al.*, 1978); the virion RNA is therefore of positive polarity and can function directly as mRNA without the requirement for virion enzymes or other proteins. In infected cells, up to seven RNA species with the characteristics of mRNAs have been identified in association with polysomes. The largest is of genome length; the other six are subgenomic RNAs which form a 'nested set' as originally found for the avian coronavirus, infectious bronchitis virus (IBV) (Stern & Kennedy, 1980) in which the sequence of each RNA is contained within the sequences of all larger RNAs, extending inward from the 3′-terminus of genome RNA (Cheley *et al.*, 1981; Lai *et al.*, 1981; Leibowitz & Weiss, 1981; Spaan *et al.*, 1982). There is evidence that these RNAs are functionally monocistronic (Siddell *et al.*, 1980, 1981) with only the 5′-terminal portion available for translation by ribosomes. Ultraviolet transcription-mapping studies of murine coronavirus-infected cells reveal that the target size of each mRNA corresponds to the size of the RNA itself, which appears to rule out the possibility that they are derived by cleavage of a full-length precursor molecule (Jacobs *et al.*, 1981). However, recent evidence indicates that each mRNA may possess a short common 'leader' nucleotide sequence at the 5′ terminus (Lai *et al.*, 1982; Spaan *et al.*, 1982), so that a novel priming or splicing mechanism may be involved in their synthesis.

This RNA synthetic mechanism is of considerable interest. It was recently reported that IBV replication requires the host cell nucleus and host transcriptional factors (Evans & Simpson, 1980); virus replication was inhibited in enucleated cells, and in the presence of α-amanitin, a drug which specifically inhibits the activity of cellular DNA-dependent RNA polymerase II, responsible for the synthesis of cellular mRNA. Although these data suggest that avian coronaviruses may share with influenza virus (Krug, 1981) a close dependence on the cell nucleus, murine coronavirus replication has been observed in enucleated cells (Wilhelmsen *et*

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al., 1981; Brayton *et al.*, 1981). As there are many biochemical features of avian and murine coronaviruses which suggest a common mode of replication (Mahy, 1981) it is important to resolve this discrepancy. In this paper we present evidence that DNA-dependent RNA transcription is not required for murine coronavirus replication, and describe the induction of an RNA-dependent RNA polymerase in murine coronavirus-infected cells. A preliminary account of this work was presented at the 5th International Congress of Virology, Strasbourg, 1981.

METHODS

Viruses and cells. Mouse hepatitis virus strains A59 and JHM were obtained and propagated on monolayer cultures of Sac(−) cells as described previously (Wege *et al.*, 1978, 1979, 1981; Siddell *et al.*, 1980). Virus infectivity was assayed by titration of the virus-induced cytopathic effect in monolayer cultures of mouse L cells ($TCID_{50}$). The cells were grown in microtitre plates (96 × 0·2 ml) and 12 wells were inoculated with each virus dilution. The titrations were incubated for 2 days at 37 °C before scoring. Analysis of RNA synthesis and polymerase induction was carried out using Sac(−) cells grown in suspension culture in spinner medium (Siddell *et al.*, 1980). For infection, the cells were gently pelleted (1000 *g* for 10 min) and resuspended to a concentration of 10^7 cells/ml in a virus suspension (tissue culture supernatant) diluted to an input multiplicity of 5 to 10 $TCID_{50}$/cell. After adsorption for 45 min at 37 °C, the cells were washed once in medium then diluted into fresh medium to a final concentration of 10^6 cells/ml.

Intracellular RNA synthesis. To measure virus-specific RNA synthesis, 5 ml amounts, containing 5 × 10^6 infected or uninfected cells, were treated at 37 °C with actinomycin D (10 μg/ml) for 30 min, then 10 μCi/ml [5,6-^{3}H]uridine (sp. act. 40 Ci/mmol) was added in the continued presence of 10 μg/ml actinomycin D. After 30 min, the cells were pelleted by centrifugation at 1000 *g* for 5 min, suspended in 1 ml NTE buffer (0·1 M-NaCl, 0·01 M-Tris–HCl pH 7·4, 0·001 M-EDTA), then snap frozen in a CO_2–ethanol bath and stored at −20 °C until assayed for acid-insoluble radioactivity.

Cell fractionation. For cell fractionation, approx. 10^8 cells were pelleted by centrifugation for 5 min at 1000 *g*. All subsequent steps were carried out at 0 to 4 °C. The cells were washed by resuspension in 10 ml NTE buffer and pelleted as before, then resuspended at 2·5 × 10^7 cells/ml in TN buffer (0·01 M-Tris–HCl pH 7·4, 0·1 M-NaCl, containing 500 Kallikrein inhibitor units/ml aprotinin), and lysed by 20 strokes of a tight-fitting Dounce homogenizer. The nuclei were separated by centrifugation at 1000 *g* for 5 min, then the post-nuclear supernatant was centrifuged at 5000 *g* for 10 min in an Eppendorf microfuge to sediment the large-particle fraction of the cytoplasm.

RNA-dependent RNA polymerase assay. Cell fractions were suspended in TN buffer to a final protein concentration of 3 to 5 mg/ml and 50 μl amounts assayed for RNA-dependent RNA polymerase activity in a standard reaction mixture containing, in a final total volume of 125 μl: 50 mM-HEPES buffer pH 8·0, 8 mM-magnesium acetate, 5 mM-sodium phospho*enol*pyruvate, 50 μg/ml pyruvate kinase, 10 mM-dithiothreitol, 500 units/ml aprotinin, 40 μg/ml actinomycin D, and 0·6 mM each of three unlabelled nucleoside triphosphates (ATP, CTP and UTP; or ATP, CTP and GTP; or CTP, GTP and UTP) with 10 μM of the fourth, tritium-labelled nucleoside triphosphate (either [^{3}H]GTP, or [^{3}H]UTP, or [^{3}H]ATP respectively, each at 20 μCi/μmol). The mixtures were routinely reacted at 37 °C for 30 min before determination of acid-insoluble radioactivity.

DNA-dependent RNA polymerase assay. The DNA-dependent RNA polymerase activity of cell nuclei was assayed in the presence of high salt and with Mn^{2+} as the divalent cation; these conditions detect the activity of the α-amanitin-sensitive DNA-dependent RNA polymerase form II (for review, see Mahy *et al.*, 1975). The nuclear fraction of Sac(−) cells was added in 50 μl amounts to a reaction mixture (final total vol. 125 μl) which consisted of 50 mM-HEPES buffer pH 8·0, 4 mM-manganese chloride, 100 mM-sodium chloride, 300 mM-ammonium sulphate, 25 mM-phospho*enol*pyruvate, 50 μg/ml pyruvate kinase, 0·6 mM each of unlabelled nucleoside triphosphate (ATP, CTP and UTP), 0·01 mM-GTP and 10 μM-[^{3}H]GTP. The assays were incubated for 30 min at 37 °C before determination of acid-insoluble radioactivity.

Determination of acid-insoluble radioactivity. RNA polymerase reactions (0·125 ml) were stopped by addition of 0·5 ml ice-cold 10% trichloroacetic acid containing 0·125 M-sodium pyrophosphate. After 10 min at 0 °C, the acid precipitates were collected on to Whatman GF/C filters which were washed in 15 ml 5% trichloroacetic acid, once in 5 ml ethanol, then dried, and acid-insoluble radioactivity was counted in toluene-based scintillation fluid in a Beckman scintillation counter. Cell pellets were suspended in 1 ml NTE buffer, precipitated by addition of 5 ml 10% trichloroacetic acid, then filtered, washed and counted as described.

Materials. α-Amanitin, phospho*enol*pyruvate and pyruvate kinase were obtained from Boehringer, Mannheim; anisomycin, aprotinin and unlabelled nucleoside triphosphates were obtained from Sigma; [^{3}H]uridine (40 Ci/mmol), [8-^{3}H]adenosine triphosphate (20 Ci/mmol), [^{3}H]guanosine triphosphate (11 Ci/mmol) and [5,6-^{3}H]uridine triphosphate (42 Ci/mmol) were obtained from Amersham International.

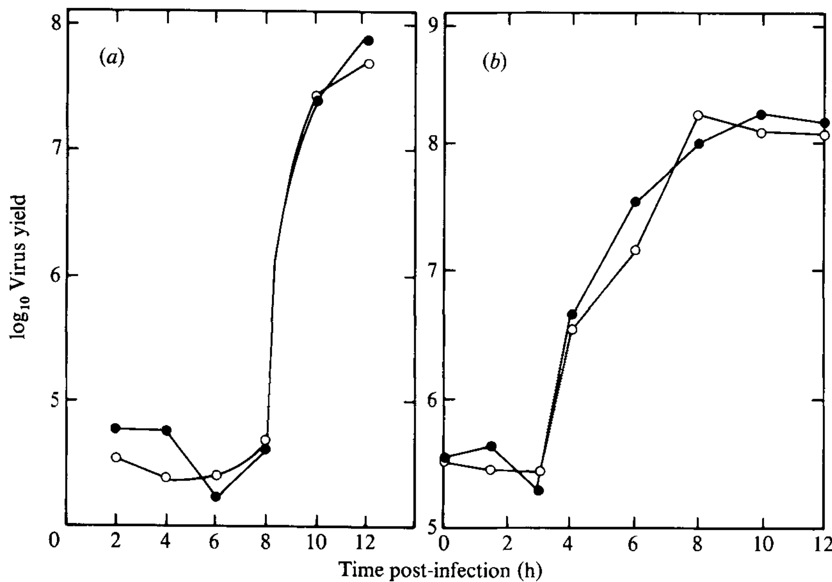

Fig. 1. Effect of α-amanitin on growth of murine coronaviruses. Sac(−) cells were infected with A59 virus (*b*) or JHM virus (*a*) in the presence (○) or absence (●) of 20 μg/ml α-amanitin as described in the text. Samples of the medium taken at intervals were titrated for infectivity on monolayer cultures of L cells.

RESULTS

Effects of α-amanitin on murine coronavirus growth

We have previously shown that murine coronavirus-specific RNA synthesis can be detected in cells treated with actinomycin D (Wege *et al.*, 1981). However, in view of the report that the type-specific coronavirus, IBV, requires host cell nuclear transcription factors for replication (Evans & Simpson, 1980), we tested the effects on murine coronavirus replication of α-amanitin, a specific inhibitor of host cell DNA-dependent RNA polymerase form II activity (Fiume & Wieland, 1970).

Since the action of α-amanitin is known to vary somewhat in different cell types, we first tested the drug on Sac(−) cells, using a procedure similar to that previously employed with chick cells (Hastie & Mahy, 1973). Batches of cells were exposed to 20 μg/ml α-amanitin for 1 h at 37 °C, then the cells were fractionated as described in Methods. The nuclear fraction was further purified by centrifugation through a 24% sucrose cushion at 1000 *g* for 10 min, to remove any traces of unbound α-amanitin, then assayed *in vitro* for DNA-dependent RNA polymerase activity using a high salt, Mn^{2+}-stimulated assay as described in Methods. The RNA polymerase activity of nuclei isolated from α-amanitin-treated cells was inhibited by more than 90% in these experiments, showing that the drug is taken up by Sac(−) cells, and is an effective inhibitor of the host cell RNA polymerase form II (data not shown). Viable cell counts of Sac(−) cell suspension cultures grown in the presence of 20 μg/ml α-amanitin gave no evidence that the drug was cytotoxic over a 24 h period. We therefore examined the effects of 20 μg/ml α-amanitin on the growth cycle in Sac(−) cells of two strains of murine coronavirus, A59 and JHM.

Sac(−) cells, either untreated or exposed to 20 μg/ml α-amanitin for 1 h, were infected with A59 or JHM virus so as to provide an input multiplicity of 5 $TCID_{50}$/cell. α-Amanitin (20 μg/ml) was added to the virus suspension where appropriate, and after adsorption the cells were diluted into fresh normal growth medium, or medium containing 20 μg/ml α-amanitin. At intervals, 1 ml samples of the medium were withdrawn, the cells removed by centrifugation, and the medium stored at −70 °C. When all the samples were collected, infectivity was titrated on monolayer cultures of L cells as described in Methods. The presence of 20 μg/ml α-amanitin during the 12 h single-cycle growth period did not significantly affect the growth of either A59 virus, or the slower-growing neurotropic strain, JHM (Fig. 1).

Table 1. *Effect of various concentrations of α-amanitin on the 24 h yield of JHM virus from Sac(−) cultures*

Concn. of α-amanitin (μg/ml)*	24 h yield of JHM virus ($TCID_{50}$/ml)
0	$1·7 \times 10^7$
5	$3·1 \times 10^7$
10	$7·2 \times 10^6$
20	$5·6 \times 10^6$
30	$4·7 \times 10^6$
40	$4·4 \times 10^6$
50	$3·5 \times 10^6$

* α-Amanitin was present at the indicated concentration in the cell medium from 1 h before infection, during virus adsorption, and throughout the 24 h period of virus growth.

We also tested the effects of a range of α-amanitin concentrations on the 24 h yield of JHM virus, again pretreating the cells for 1 h before infection, and maintaining the appropriate α-amanitin concentrations throughout the 24 h period (Table 1). Under these conditions, a small but definite decrease in total yield was observed at α-amanitin concentrations greater than 10 μg/ml. However, even in the presence of 50 μg/ml α-amanitin, which caused cytopathic effects in uninfected cells which were visible by phase-contrast microscopy, the total yield was only decreased by 0·7 $\log_{10}$.

From these results we conclude that α-amanitin has no specific inhibitory effects, and that murine coronavirus replication does not require the participation of host cellular mRNA synthesis.

Effect of anisomycin on virus-specific RNA synthesis

We next determined whether new protein synthesis was required for the replication of murine coronavirus RNA, as would be expected if a new virus RNA-dependent RNA polymerase is induced in infected cells. For these and subsequent experiments we used the A59 virus strain, which induces a high rate of virus-specific RNA synthesis in Sac(−) cells, detectable by pulse-labelling with [^{3}H]uridine in the presence of actinomycin D to shut down host cell RNA synthesis. We chose anisomycin as the protein synthesis inhibitor in these experiments since it has been found more effective than cycloheximide (Stillman *et al.*, 1981). When Sac(−) cells were infected with A59 virus and pulse-labelled at intervals for 30 min with [^{3}H]uridine in the presence of actinomycin D (10 μg/ml) the maximum rate of RNA synthesis was observed at 5 h post-infection (see Fig. 5). In different experiments, a 30- to 50-fold stimulation of [^{3}H]uridine incorporation was observed at this time in A59 virus-infected compared to uninfected Sac(−) cells.

To test the effects of protein synthesis inhibition, aliquots of infected cells were pulse-labelled with [^{3}H]uridine from 5 to 5·5 h post-infection after addition of anisomycin (10 μM) at various times from 0 to 5 h post-infection. In the same experiment the rate of RNA synthesis in infected and uninfected cells not treated with anisomycin was also measured by pulse-labelling at various times up to 5 h post-infection (Fig. 2). Virus-specific RNA synthesis was sensitive to anisomycin added at any time from 1 h post-infection, and even when added at 5 h, caused a 50% decrease in [^{3}H]uridine incorporation. In other experiments (data not shown) cells were infected for 5 h, then 10 μM-anisomycin was added and the cells were pulse-labelled at 15 min intervals; virus-specific RNA synthesis was inhibited by 50% within 15 min, suggesting that a labile or continuously synthesized protein is necessary for A59 virus-specific RNA synthesis.

RNA-dependent RNA polymerase activity in infected cells

Since the maximum rate of actinomycin D-resistant RNA synthesis was observed in cells at 5 h after A59 virus infection, we fractionated batches of cells at this time and assayed for RNA-dependent RNA polymerase activity *in vitro* (Table 2). We included the protein cleavage inhibitor aprotinin in the buffers during cell fractionation and polymerase assay, in view of the possibility revealed above that the enzyme was a labile protein. As shown in Table 2, RNA

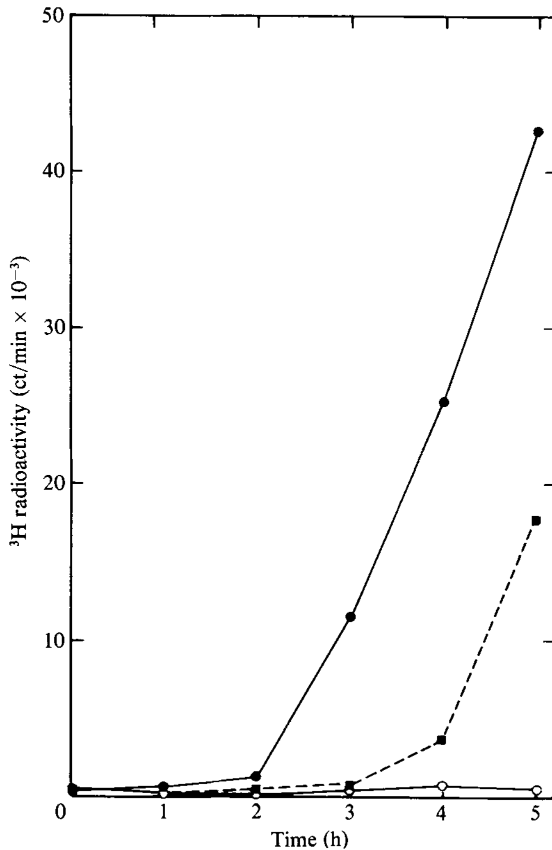

Fig. 2

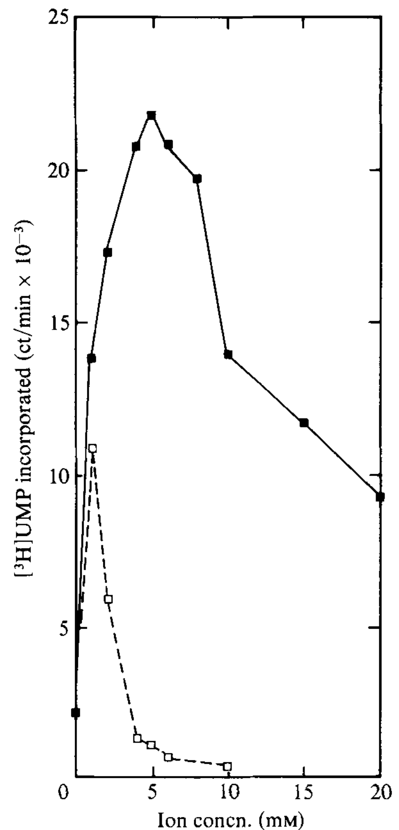

Fig. 3

Fig. 2. Effect of inhibition of protein synthesis on A59 virus-specific RNA synthesis. Sac(−) cells were infected with A59 virus and at intervals, aliquots containing 5 × 10^6 infected (●) or uninfected (○) cells were treated for 30 min with actinomycin D (10 μg/ml), then [^{3}H]uridine was added for a further 30 min in the continued presence of actinomycin D before determination of incorporation into acid-insoluble radioactivity. The points indicate the commencement of the [^{3}H]uridine pulse-label. To parallel samples of infected cells, anisomycin (10 μM) was added at the times indicated (■), then at 5 h each sample was pulse-labelled with [^{3}H]uridine in the presence of actinomycin D as with the 5 h infected and uninfected cells.

Fig. 3. Effect of varying concentrations of Mg^{2+} (■) or Mn^{2+} (□) on RNA-dependent RNA polymerase activity.

polymerase activity was detectable even in lysed whole cells in the presence of actinomycin D *in vitro* to inhibit the host cellular DNA-dependent RNA polymerase. The RNA-dependent polymerase activity was also found in the post-nuclear supernatant of infected cells, where it was associated with the large particle, 10000 *g* pellet, fraction (Table 2). The data shown in Table 2 are representative of experiments using cell fractions obtained from cultures where the stimulation of RNA synthesis *in vivo* as measured by [^{3}H]uridine incorporation in the presence of 10 μg/ml actinomycin D was at least 50-fold higher in infected compared to uninfected cells. In experiments where *in vivo* RNA synthesis was stimulated less than 25-fold the demonstration of *in vitro* RNA-dependent RNA polymerase activity was unconvincing.

Optimum reaction conditions in vitro

The post-nuclear supernatant fraction from cells infected for 5 h with A59 virus was used to establish optimum requirements for *in vitro* polymerase activity.

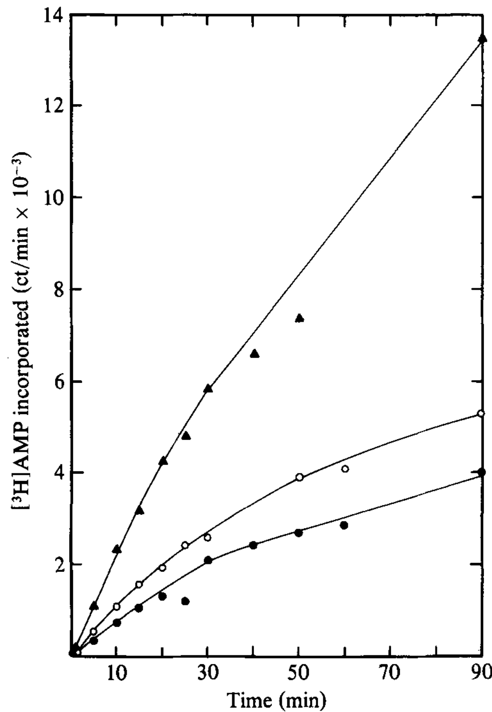

Fig. 4. Kinetics of *in vitro* RNA synthesis catalysed by the post-nuclear supernatant of 5 h A59 virus-infected cells at 37 °C (▲), 31 °C (○) or 28 °C (●).

Table 2. *RNA-dependent RNA polymerase activity in A59 virus-infected Sac(−) cells at 5 h post-infection*

Fraction	Ct/min [3H]GMP incorporated/mg protein/60 min: Uninfected	Infected	Fold Stimulation
Dounced cells	6830	59990	8·8
Dounced cells assayed without actinomycin D	82118	79260	—
Post-nuclear supernatant	4990	53854	10·8
10000 *g* pellet	5234	47090	9·0
10000 *g* supernatant	4166	11038	2·6

A divalent cation was necessary for polymerase activity; Mg^{2+} at 5 mM gave maximal incorporation. About 50% of the activity observed with Mg^{2+} was obtained in the presence of 1 mM-Mn^{2+}, but higher concentrations were inhibitory (Fig. 3). No activity was observed in the presence of Ca^{2+} at 1 mM or 5 mM.

The time course of the enzyme reaction at three temperatures is presented in Fig. 4. The lower temperatures of 31 °C and 28 °C were chosen since they have been found optimum for influenza virion RNA transcriptase and influenza virus-induced RNA-dependent RNA polymerase activities respectively (Mahy *et al.*, 1975).

The coronavirus-induced RNA polymerase reaction proceeded in almost linear fashion for at least 90 min *in vitro* at 37 °C and at much slower rates at 28 °C or 31 °C (Fig. 4).

Other experiments (data not shown) established that the enzyme activity was optimal over a fairly broad pH range (pH 8·0 to pH 8·4), and was independent of total unlabelled nucleoside triphosphate concentration above a level of 0·5 mM in the reaction mixture.

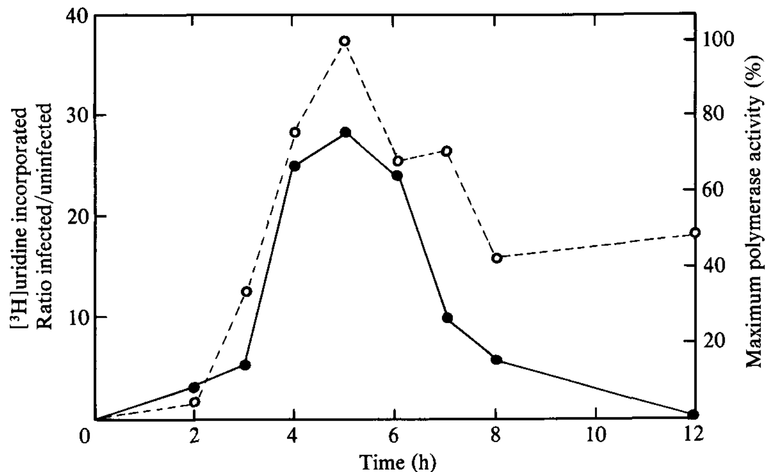

Fig. 5. Comparison of *in vivo* and *in vitro* RNA synthesis. A59 virus-infected or uninfected Sac(−) cells were assayed at intervals for actinomycin D-resistant [^{3}H]uridine incorporation *in vivo*, and the infected/uninfected ratio determined (●). At the same time, infected cells were fractionated and the post-nuclear supernatant was assayed *in vitro* for RNA-dependent RNA polymerase activity (○). The results of these assays are expressed as the percentage of the maximum activity, which occurred at 5 h post-infection.

Time course of induction of RNA-dependent RNA polymerase activity in vivo

RNA polymerase activity was assayed using the post-nuclear supernatant fractions from batches of cells harvested at intervals following infection. Thirty min before each time point 2×10^7 infected or uninfected cells were treated with actinomycin D (10 μg/ml) for 30 min, then 10^7 cells were fractionated for determination of RNA polymerase activity. The other 10^7 cells were pulse-labelled with [^{3}H]uridine for 30 min, then analysed for acid-insoluble radioactivity. The results showed that the peak of both *in vivo* and *in vitro* RNA synthesis assays occurred at 5 h post-infection (Fig. 5). The coincidence between the two activities suggests that the RNA polymerase activity detected *in vitro* is responsible for the synthesis of RNA as measured *in vivo*. Polymerase activity could still be detected in appreciable amounts up to 12 h post-infection, when [^{3}H]uridine incorporation had dropped to the control uninfected level, presumably due to the extensive cytopathic effects observable by phase-contrast microscopy of 12 h-infected cells.

DISCUSSION

Our data suggest that DNA-directed RNA synthesis, which is sensitive to α-amanitin or actinomycin D, plays no significant role in murine coronavirus replication. If, as has been reported, IBV requires host cell RNA synthesis for replication (Evans & Simpson, 1980), there might be fundamental differences in the replication strategies of avian and murine coronaviruses. Further investigation of the avian coronavirus dependence on host cell DNA transcription may help to explain these differences. Since our experiments were carried out, two other laboratories have reported that murine coronaviruses can replicate in enucleated host cells, and in the presence of inhibitors of cell nuclear function (Wilhelmson *et al.*, 1981; Brayton *et al.*, 1981). These reports and our own results appear to exclude participation of cellular DNA transcription in murine coronavirus replication and suggest that the mechanism by which coronavirus mRNAs are formed differs fundamentally from that involved in influenza virus mRNA production, where host nuclear functions are essential (Krug, 1981).

The coincidence of the peaks of RNA synthesis as measured *in vivo* and *in vitro* leave little doubt that the RNA-dependent RNA polymerase activity which we describe is responsible for the bulk of virus-specific RNA synthesis in murine coronavirus-infected cells. The enzyme activity was primarily located in the large particle fraction of the cytoplasm. The only previous report of a coronavirus-induced RNA polymerase concerned porcine transmissible gastroenteri-

tis virus infection of a swine testicle cell line (Dennis & Brian, 1981, 1982) and in this case also the activity was found in cytoplasmic membrane-associated complexes. Several other characteristics of this enzyme activity, such as the Mg^{2+} optimum, appear similar to those which we observed with the murine virus. The coincidence between the time of maximum appearance of RNA polymerase activity (5 h) and the highest level of actinomycin D-resistant RNA synthesis in A59 virus-infected cells strongly suggests that the bulk of murine coronavirus-specific RNA is made by an RNA-dependent enzyme similar to that observed in cells infected with other positive-strand viruses such as alphaviruses (Ranki & Kaariainen, 1979). At present, there have been no virus-specific proteins identified *in vivo* or *in vitro* which would appear as candidates for the coronavirus-induced enzyme. However, no translation product has yet been identified from the 5′-terminal 'A' gene of virion RNA (Siddell *et al.*, 1982) which can encode a protein of 200000 mol. wt. and which, by analogy with alphaviruses (Strauss & Strauss, 1977), might be expected to function as a polymerase component.

We found that the coronavirus-induced RNA polymerase activity was labile in Sac(−) cells, and decreased rapidly in the presence of protein synthesis inhibitors such as anisomycin, or cycloheximide (B. W. J. Mahy *et al.*, unpublished data). We also found it necessary to assay for polymerase activity *in vitro* immediately after cell disruption. Therefore, for further investigation of the enzyme, and in particular, studies on the nature of the product RNA synthesized *in vitro* and the polypeptide components of the enzyme, it will be necessary to develop a means of stabilizing the activity.

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