

The Coronavirus Avian Infectious Bronchitis Virus Requires the Cell Nucleus and Host Transcriptional Factors

MARTIN R. EVANS AND ROBERT W. SIMPSON¹

*Waksman Institute of Microbiology, Rutgers—The State University, P.O. Box 759,
Piscataway, New Jersey 08854*

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Replication of avian infectious bronchitis virus in permissive BHK-21 cells is blocked when these cells are enucleated or irradiated with ultraviolet light prior to infection, or if cells are treated with α -amanitin during the virus growth cycle. This coronavirus, like influenza virus, can replicate normally in the presence of α -amanitin in Chinese hamster ovary cells which possess a drug-resistant RNA polymerase II. These findings indicate that avian infectious bronchitis virus requires the intact cell nucleus and one or more host transcriptional functions for productive infections. Preliminary data suggest that these cellular functions involve some aspect of virus-directed RNA synthesis.

INTRODUCTION

Various members of the coronavirus group have recently been shown to contain single-stranded RNA genomes of positive messenger polarity (Schochetman, *et al.*, 1977; Lomniczi and Kennedy, 1977; Lai and Stohman, 1978). Since it has not been firmly established whether the replication of the prototype coronavirus, avian infectious bronchitis virus (IBV), is restricted to cytoplasmic sites and whether productive infections with this ribovirus are regulated, in part, by unidentified host factors (McIntosh, 1974; Tyrrell *et al.*, 1978), we undertook the present investigation of IBV biogenesis utilizing inhibitors which compromise normal function of the host cell transcriptional apparatus. During the conduct of this work, it was recently reported that a human coronavirus is blocked for infectious virus production but not virus-directed RNA synthesis in cells treated with actinomycin D (Kennedy and Johnson-Lussenberg, 1979). A similar observation has since been made and extended in our laboratory with IBV (Evans, Welsh, and Simpson, in preparation). The findings presented in this report further establish that IBV replication requires active participation of the intact cell nucleus.

We also demonstrate that this avian coronavirus resembles influenza virus in having a need for both functionally competent host cell DNA and DNA-dependent RNA polymerase II during productive infections (Mahy *et al.*, 1972; Lamb and Choppin, 1977; Spooner and Barry, 1977). However, we find that IBV differs from influenza virus in being able to stimulate uptake of RNA precursors when actinomycin D is added to BHK-21 cells at the time of infection.

MATERIALS AND METHODS

Viruses and Cells. The highly egg-adapted Beaudette strain of IBV was used throughout. Preparation of egg-grown stocks of IBV has been previously described (Schochetman *et al.*, 1977) and in some cases eggs were incubated for only 16-18 hr. Working stocks of influenza A virus (WSN strain) were prepared by allantoic infection of 10-day-old white leghorn embryonated chicken eggs. Vesicular stomatitis virus (VSV, Indiana strain) and herpes simplex virus type 2 (HSV-2) were grown in BHK-21 cells for 16 hr at 37° using reinforced MEM (RMEM) medium (Bablanian *et al.*, 1965) containing 10% heat-inactivated fetal calf serum (FCS) and 10% tryptose phosphate broth (TPB). BHK-21 (clone 13) cells obtained from the American Type Culture Collection

¹ To whom reprint requests should be addressed.

were grown as subconfluent monolayers in RMEM with 10% FCS in 60-mm petri dish cultures for use in plaque assays or viral growth experiments as described below. Lines of α -amanitin-sensitive (CHO-S) and resistant (CHO-R) Chinese hamster ovary cells that were originally isolated in the laboratory of Dr. Lou Siminovitch (Chan *et al.*, 1972) were provided by Dr. Purnell Choppin. These lines were maintained in RMEM containing 10% FCS and supplementary proline (11.5 $\mu\text{g}/\text{ml}$). MDCK cells, used for plaque assay of WSN influenza virus, were originally obtained from Dr. Robert Krug and maintained in RMEM containing 10% FCS.

Plaque assays and egg infectivity titrations. We ("Abstr. 4th Int. Congr. Virol.," No. 34, p. 450, 1978) have recently demonstrated that the Beaudette strain of IBV but not other strains grows productively and produces plaques in BHK-21 cells despite an earlier report that these cells are nonpermissive for this avian coronavirus (Yamada *et al.*, 1971). Otsuki *et al.*, (1979) also have found BHK-21 cells to support IBV-Beaudette replication. For plaque assays, 0.2 ml aliquots of 10-fold virus dilutions made in balanced salt solution (BSS) containing 1% gelatin (GBSS) (Schochetman *et al.*, 1977) were inoculated into subconfluent monolayer cultures of BHK-21 cells. Virus adsorption was carried out for 60 min at room temperature. Each plate received 5-ml aliquots of Eagle's MEM containing 2% FCS, 4% TPB, and 0.6% agarose (Seakem, Rockland, Maine). Certain lots of fetal calf serum were found to contain factors inhibitory for IBV plaque production making it necessary to screen serum from commercial sources for its suitability. Plates were incubated for 4 days at 35° in a humidified atmosphere of 8% CO₂/air. Titrations of WSN virus were performed in MDCK monolayer cultures incubated for 3 days at 37° and serum was omitted from the overlay. Plaques were visualized by subsequent addition of MEM overlay containing neutral red at a final concentration of 0.003%. Egg infectivity titrations were performed as previously described (Schochetman *et al.*, 1977).

Infection of enucleated cells. The technique for enucleation of cells was fashioned

after the method of Carter (1967) and modified according to our specific needs. Subconfluent sheets of BHK-21 cells established on the walls of glass Corex tubes (17 × 100 mm) were washed free of growth medium with BSS. Prewarmed (37°) aliquots of RMEM containing 10 $\mu\text{g}/\text{ml}$ of cytochalasin B were added to tubes which were immediately centrifuged in a prewarmed (37°) rotor at 9500 *g* at 37° for 30 min. After centrifugation, the drug-containing medium was replaced with regular RMEM containing 10% FCS and the cultures were held at room temperature until the cells regained an approximate normal morphology in about 30 min. Control cultures were treated similarly except that they were either not centrifuged after drug treatment or they were centrifuged without subsequent drug treatment. The degree of enucleation was estimated by staining drug-treated cultures with hematoxylin and eosin and counting the number of nucleated cells among a total of about 300 cells. The number of cells per culture was estimated by counting trypsin-dispersed cells with the aid of a hemocytometer. Treated and untreated BHK-21 cultures were infected with IBV at an input multiplicity of about 60 egg infectious units (EIU) per cell. After adsorption of virus for 30 min at 35°, the cells were washed twice with BSS before addition of RMEM containing 2% FCS and 4% TPB. Cultures were incubated at 35° for 24 hr postadsorption. All cultures were frozen-thawed, and the resulting lysates were clarified and assayed for virus content by plaquing on BHK-21 cells. As a control, the same procedure described above was followed using VSV for infection.

Determination of [³H]uridine incorporation. Subconfluent BHK-21 cell monolayers in 60-mm petri dishes were exposed to IBV-Beaudette at an input multiplicity of 50–60 EIU/cell for 30 min. Infected cultures received 5 ml of RMEM (with 2% FCS and 4% TPB) and α -amanitin (20 $\mu\text{g}/\text{ml}$) was added to one group of cultures immediately after adsorption (0 time) and to another group after cultures had been first incubated at 37° for 2 hr postadsorption. For each of these groups, after cells had been incubated with α -amanitin for 3 hr, the medium was replaced with RMEM containing actinomycin

D (0.75 $\mu\text{g/ml}$) and 1 hr later all cultures received 25 μCi per plate [^3H]uridine. Thereafter, these cell monolayers were pulse-labeled for 2 hr at 37° before being washed three times with cold BSS, removed by scraping, and sedimented by low-speed centrifugation. To the packed cells was added 3 ml of cold 20% trichloroacetic acid (TCA) and the precipitates formed after overnight incubation at 4° were collected on Schleicher-Schuell B-6 cellulose nitrate filters. The precipitates were washed five times with 5-ml aliquots of cold 5% TCA and once with cold 95% ethanol. After being dried, the filters were incubated for 2 hr at room temperature in liquid scintillation vials containing 0.2 ml H₂O and 1 ml Protosol (New England Nuclear, Boston, Mass.) before further 1-ml additions of benzoyl peroxide (ICN Pharmaceuticals, Plainview, N. Y.) and Protosol were made. Vials were agitated mechanically for 60 min after which 10 ml of a toluene-based cocktail (4.0 g PPO, 0.2 g dimethyl-POPOP/liter toluene) containing 1% ascorbic acid was added. The vials were counted for radioactivity in a Beckman LS-8000 scintillation counter using external tritium standardization.

Chemicals. The α -amanitin was obtained from Sigma Chemical Co. (St. Louis, Mo.). Actinomycin D was a gift from Dr. J. Oliver Lampen of this Institute. Cytochalasin B was obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wis.). The [^3H]uridine (specific activity = 27.9 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.).

RESULTS

IBV Infection of Enucleated BHK-21 Cells.

Previous studies on the capacity of enucleated cells to support replication of various animal viruses have excluded an analysis of representative coronaviruses although other positive-stranded viruses were tested (Follett *et al.*, 1975). Picornaviruses, alphaviruses, flaviviruses, and bunyaviruses have not exhibited a strict requirement for either nuclear factors or cellular transcription (Follett *et al.*, 1975; Obijeski and Murphy, 1977; Goldman *et al.*, 1977). In order to establish the importance of host nuclear func-

TABLE 1
REPLICATION OF IBV IN ENUCLEATED
BHK-21 CELLS^a

Treatment of cells	24-hr virus yield expressed as PFU/cell
Centrifugation and IBV infection	0.009
Cytochalasin/centrifugation and IBV infection	<0.00005
IBV infection only	0.007
Cytochalasin and IBV infection	0.008
Cytochalasin/centrifugation and VSV infection	2000
Centrifugation and VSV infection	6740
Cytochalasin and VSV infection	5100

^a Sheets of BHK-21 cells attached to the walls of glass Corex tubes (17 × 100 mm) were exposed to 10 $\mu\text{g/ml}$ of cytochalasin B while being centrifuged at 9500 *g* as described under Materials and Methods. The treated cells were infected with either IBV (70 EIU per cell) or VSV-Indiana (10 PFU/cell). The infected cells were incubated with RMEM medium at 35° for 16 (VSV) or 24 (IBV) hr after which they were titrated for virus content by plaque assay in normal BHK-21 cells. Controls included cells that were only centrifuged or only treated with cytochalasin prior to infection. Combined drug treatment and centrifugation resulted in enucleation of >95% of the attached cells.

tions for the IBV replication cycle, BHK-21 cells enucleated by treatment with cytochalasin B (see Materials and Methods) were tested for their permissiveness. The inability of cytoplasts derived from these cells to support replication of IBV-Beaudette is illustrated by the data presented in Table 1. Since an appreciable number of cells were detached from the culture vessels as a result of centrifugation, virus titers are more accurately expressed as the number of infectious particles (PFU) per cell. Control cells exposed to cytochalasin without centrifugation showed a slight reduction of their virus-producing capacity indicating that the drug did not exert a strong cytotoxic effect on BHK-21 cells. In contrast to the results obtained with IBV, replication of the rhabdovirus, VSV, in enucleated BHK-21 cells was nearly as efficient as with infections carried out in intact cells. The resulting two- to three-fold reduction in PFU

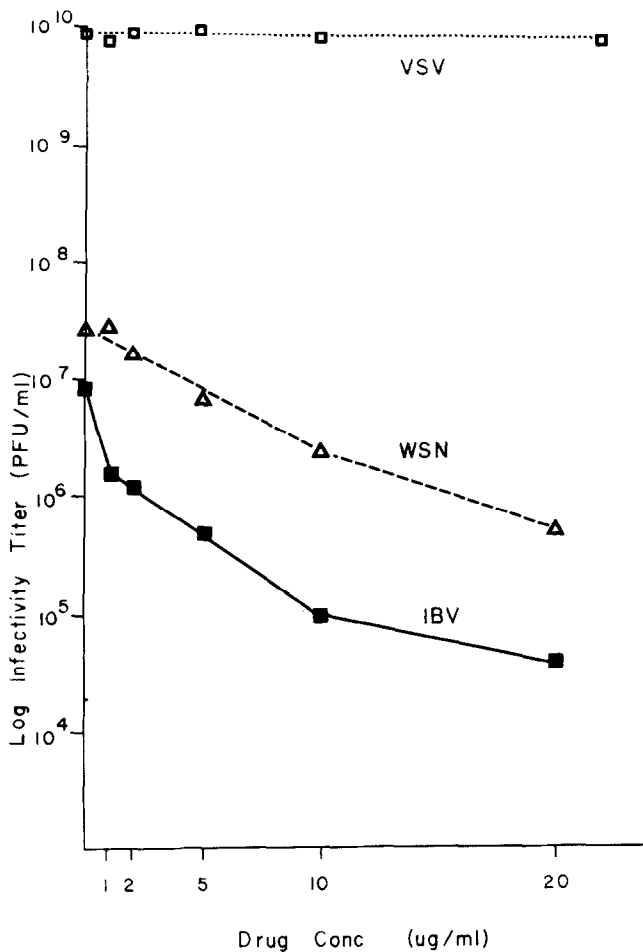


FIG. 1. Growth of IBV in α -amanitin-treated cells. Subconfluent monolayers of BHK-21 cells were infected with IBV (50 EIU/cell). Following virus adsorption at 37° (60 min), the infected cells were incubated for 24 hr at 37° with either regular RMEM or medium containing various concentrations of α -amanitin. The 24-hr virus yields were determined by standard plaque assay in BHK-21 cells. Controls included cultures originally infected with VSV (2 PFU/cell) or WSN virus (2 PFU/cell). Infection with IBV (solid squares); WSN (open triangles); VSV (open squares).

yield of VSV for enucleated cells compared to controls may be partly attributable to a reduction in the amount of cytoplasm that has occurred during the enucleation process as described previously by other investigators (Wiktor and Koprowski, 1972).

Growth of IBV in Various Cell Lines Treated with α -Amanitin

IBV does not replicate in enucleated cells as described above and it has also been observed that actinomycin D blocks production of human or avian coronaviruses without

impairing virus-induced uptake of RNA precursors (Kennedy and Johnson-Lussenberg, 1979; Evans, Welsh, and Simpson, in preparation). Since both enucleation and actinomycin D are also known to abort myxovirus infections (Kelly *et al.*, 1974; Barry, 1964; Rott *et al.*, 1965; Mark *et al.*, 1979), we wished to determine whether IBV, like influenza virus, might require the function of specific nuclear RNA polymerases for its growth cycle in permissive cells (Mahy *et al.*, 1972; Lamb and Choppin, 1977; Spooner and Barry, 1977). IBV was grown in α -

amanitin-treated BHK-21 cells for 24 hr as described in Fig. 1. At a drug concentration of 20 $\mu\text{g/ml}$, titers of IBV were 0.03% of those of the untreated infected control cultures. WSN influenza virus was also strongly inhibited by α -amanitin under the test conditions employed whereas VSV, a cytoplasmic ribovirus, was insensitive to α -amanitin over the concentration range employed. (Fig. 1).

The finding that productive infections of BHK-21 cells with IBV are aborted by α -amanitin suggested that nuclear RNA polymerase II plays an unexpectedly important role in the replication of this positive-stranded virus since it is recognized that this enzyme is preferentially inhibited by this drug at appropriate concentrations (Roeder, 1976). To demonstrate more conclusively that one or more host transcriptional functions mediated by RNA polymerase II regulate IBV infections, we compared the ability of IBV to replicate in α -amanitin-resistant mutants of CHO cells in the presence and absence of drug since these cells are known to possess an α -amanitin-resistant form of this enzyme (Lobban *et al.*, 1976). These cells enable one to determine if the action of α -amanitin is due to a direct effect on cellular RNA polymerase II rather than a viricidal action or a secondary effect on the cells. WSN influenza virus was included as a control in these experiments since earlier studies had shown that this myxovirus can productively infect CHO-R cells in the presence of α -amanitin (Lamb and Choppin, 1977; Spooner and Barry, 1977). The experiment summarized in Table 2 shows that IBV, like influenza virus, replicates almost equally well in untreated and α -amanitin-treated CHO-R cells, whereas both viruses are strongly inhibited in progenitor CHO-S cells in the presence of drug. It should be noted that peak yields of IBV-Beaudette from untreated CHO-S and CHO-R cells are substantially less than those obtained from the BHK-21 cell line, thus indicating their semi-permissive nature for this coronavirus. Collectively, these results provide evidence that normal function of cellular RNA polymerase II is required for coronavirus replication in the cell systems examined.

TABLE 2

RELATIVE YIELDS OF IBV FROM α -AMANITIN-RESISTANT AND -SENSITIVE CHO CELLS INFECTED IN THE PRESENCE OF DRUG^a

Cells and drug treatment	24-hr virus yield (PFU/ml) of cells infected with	
	IBV	WSN
Untreated CHO-S	2.7×10^3	5.0×10^5
Untreated CHO-R	4.1×10^3	9.3×10^5
CHO-S + α -amanitin	$<10^1$	6.5×10^3
CHO-R + α -amanitin	1.9×10^3	3.7×10^5

^a CHO cells that are sensitive (CHO-S) or resistant (CHO-R) to the inhibitory effects of α -amanitin were infected with either IBV or influenza WSN virus at input multiplicities of 70 EIU/cell (IBV) or 5 PFU/cell (WSN). Infected cells were incubated for 24 hr at 37° with RMEM medium in the presence or absence of 20 $\mu\text{g/ml}$ α -amanitin after which they were assayed for plaque-forming virus in either BHK-21 cells (IBV) or MDCK cells (WSN virus).

Uptake of RNA Precursors by Infected Cells Treated with α -Amanitin and Actinomycin D

The use of actinomycin D at low concentrations will inhibit cellular but not coronavirus-directed incorporation of RNA precursors (Kennedy and Johnson-Lussenberg, 1979; Evans, Welsh, and Simpson, in preparation). Furthermore, since we have found that IBV infection of BHK-21 cells does not strongly inhibit host cell RNA synthesis, it is necessary to treat cells with actinomycin D to permit monitoring of virus-specific RNA synthesis. To examine the effect of α -amanitin on viral RNA synthesis, BHK-21 cells were exposed for 3 hr to α -amanitin either immediately after virus adsorption (0 time) or 2 hr later. As shown in Table 3, infected cells treated only with actinomycin D gave twofold higher incorporation levels than the uninfected controls reflecting virus-specific RNA synthesis. If only α -amanitin was added at 0 time or 2 hr postinfection, virus-induced [³H]uridine incorporation could not be measured since total cellular RNA synthesis is not strongly inhibited by this drug. Sequential treatment of IBV-infected cells with α -amanitin and actinomycin

TABLE 3
INCORPORATION OF [³H]URIDINE IN BHK-21 CELLS INFECTED WITH IBV IN THE PRESENCE
OF α -AMANITIN AND/OR ACTINOMYCIN D^a

Treatment of cells		Radioactivity of drug-treated cells			
		Group A		Group B	
Virus	Drug(s)	dpm ($\times 10^4$) ^b	Ratio test/control	dpm ($\times 10^4$) ^b	Ratio test/control
IBV	Actinomycin D	14.9	1.98	6.9	1.86
IBV	α -Amanitin	129.4	0.85	138.8	0.82
IBV	α -Amanitin + actinomycin D	4.7	0.98	4.5	1.03
None	Actinomycin D	7.1 ^c		4.1	
None	α -Amanitin	151.6		163.3	
None	α -Amanitin + actinomycin D	4.9		4.3	

^a Confluent monolayer cultures of BHK-21 cells were infected with IBV-Beaudette at an input of 58 EIU/cell. Cultures received RMEM medium containing α -amanitin (20 μ g/ml) either immediately after adsorption (group A) or after incubation of cultures at 37° for 2 hr postinfection (group B). Following 37° incubation of cells with α -amanitin for 3 hr, the medium was replaced with RMEM containing actinomycin D (0.75 μ g/ml) and 60 min later the cultures were pulse-labeled for 2 hr at 37° with 5 μ Ci/culture [³H]uridine (sp act = 27.9 Ci/mmol). Thereafter, the cells were processed for determination of acid-insoluble radioactivity as described under Materials and Methods. Control cultures received regular RMEM in place of medium containing either actinomycin D or α -amanitin at the appropriate time intervals.

^b Disintegrations per minute.

^c Uninfected cells in the presence of actinomycin D showed a 97% reduction of radioisotope incorporation by comparison with the untreated control.

D, respectively, resulted in a suppression of virus-specific RNA synthesis whether or not α -amanitin was added at 0 time or 2 hr post-infection. This finding indicates that the sensitivity of virus-directed RNA synthesis to α -amanitin is not strictly confined to the early stages of IBV replication in BHK-21 cells. It is also possible that the suppressed [³H]uridine uptake by infected cells observed in these experiments partly reflects an additive effect of the two drugs employed.

Abortive Replication of IBV in Ultraviolet-Irradiated BHK-21 Cells

We have also examined ultraviolet radiation as an inhibitor of cellular DNA function which is known to compromise replication of animal viruses such as influenza (Barry, 1964; Rott *et al.*, 1965) or vaccinia virus (Hruby *et al.*, 1979) to establish whether growth of IBV is also sensitive to these treat-

ments. BHK-21 cell monolayers exposed to a uv light source for different time intervals were infected with IBV-Beaudette and 24-hr yields were assayed for infectivity. The results of this experiment (Fig. 2) clearly show that this treatment renders BHK-21 cells nonpermissive for both IBV and WSN influenza. Conversely, we found that VSV was able to sustain productive infections in BHK-21 cells under the same conditions of irradiation with uv (data not shown).

DISCUSSION

The experimental approaches used in this investigation have demonstrated the requirement for both a functionally competent cell nucleus and cellular polymerase II in the replication of infectious bronchitis virus. First, physical removal of nuclei from BHK-21 cells by treatment with cytochalasin B rendered these cells nonpermissive for IBV

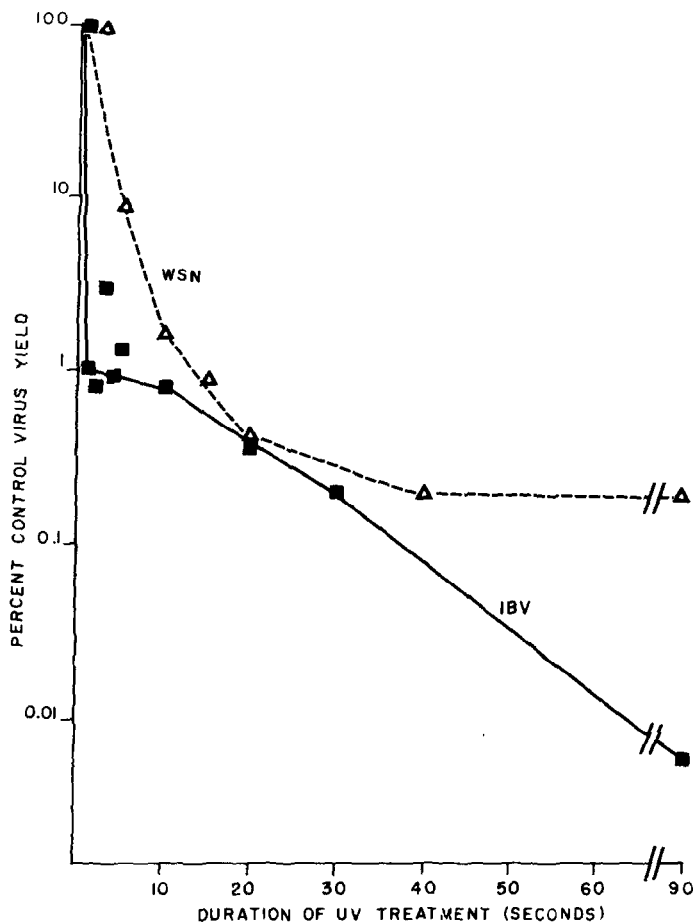


FIG. 2. Relative yields of IBV in BHK-21 cells exposed to different doses of uv light prior to infection. Subconfluent BHK-21 cell monolayers were irradiated for intervals up to 90 sec at a distance of 30 cm with the light from a 15-W germicidal lamp (General Electric, G1578 bulb) emitting $320 \mu\text{W}/\text{cm}^2$. IBV (60 EIU/cell) or WSN influenza virus (1 PFU/cell) was added to duplicate plates irradiated for the same time interval and adsorbed at 37° for 1 hr. The infected cultures were incubated at 37° for 24 hr with 5-ml aliquots of RMEM and 2% FCS. Cell lysates obtained by freezing-thawing were tested for virus content by plaque assay in either BHK-21 cells (IBV) or MDCK cells (WSN). Virus yields are expressed as percentage of the unirradiated control titers. Infection with IBV (solid squares); WSN (open triangles).

(Beaudette) without impairing their capacity to replicate the cytoplasmic ribovirus VSV. It is yet to be determined whether virus-specific macromolecular synthesis occurs in IBV-infected cytoplasts in the absence of infectious virus production. Whether such activity will be detected in enucleates infected with this positive stranded coronavirus will depend on its specific mode of replication; that is, whether or not initiation of viral RNA synthesis and early processing of viral mRNA species are nucleus-

dependent events. Work in progress aimed at understanding how IBV interacts with nuclear and cytoplasmic compartments during its biosynthesis hopefully will resolve this question.

Second, we have shown that treatment of permissive BHK-21 cells with the potent fungal toxin α -amanitin markedly inhibits productive infections both by IBV and WSN influenza virus. At low concentrations ($\sim 10 \mu\text{g}/\text{ml}$), this drug is recognized to act as a relatively specific inhibitor of DNA-depend-

ent RNA polymerase II, the nuclear enzyme of eucaryotic cells responsible for messenger RNA synthesis (Roeder, 1976). A more rigorous proof for an obligatory role of RNA polymerase II in the IBV replication cycle is found in our experiments involving the α -amanitin-resistant CHO-R mutant cell line that possesses an RNA polymerase II which is insensitive to this drug (Lobban *et al.*, 1976). We have confirmed earlier reports that influenza virus is resistant to the inhibitory effects of α -amanitin when grown in CHO-R cells (Lamb and Choppin, 1977; Spooner and Barry, 1977) and have extended these findings to the avian coronavirus, IBV. A similar approach has recently been used by Silver *et al.* (1979) for implicating RNA polymerase II as an essential host factor required for poxvirus biogenesis.

Finally, it has been possible to abort infectious virus production by IBV in BHK-21 cells irradiated with uv light prior to infection. The generation of pyrimidine dimers in nucleic acid molecules by this treatment is known to block normal host DNA replication and RNA transcription, and the uv doses employed in our experiments would be expected to strongly inhibit both DNA and RNA synthesis (cf. Hruby *et al.*, 1979). Thus, although uv irradiation simultaneously inhibits a number of cellular biosynthetic activities, it is clear that radiation-damaged BHK-21 cells with functionally impaired nuclei are unable to support active replication of infectious IBV particles.

An interesting outcome of our studies with IBV is the realization that this RNA virus grossly resembles negative-stranded myxoviruses for the sensitivity of their growth cycle to experimental conditions that compromise either the cell nucleus (Kelly *et al.*, 1974), normal DNA function (Barry, 1964; Rott *et al.*, 1965), or nuclear RNA polymerase II activity (Mahy *et al.*, 1972; Lamb and Choppin, 1977; Spooner and Barry, 1977). It is already quite apparent, however, that these two disparate classes of animal riboviruses differ at the molecular level in their requirements for specific host factors during the viral replication cycle. We have found that inhibitors of functional cellular DNA such as actinomycin D or mito-

mycin C strongly block production of infectious IBV progeny in BHK-21 cells (Evans, Welsh, and Simpson, in preparation). Unlike influenza virus, however, the growth of IBV remains sensitive to actinomycin D relatively late in the infection, while throughout the time course this drug has no appreciable effect on virus-induced sustained uptake of radiolabeled RNA precursors. Kennedy and Johnson-Lussenburg (1979) have observed a similar effect of actinomycin D on the biogenesis of human coronavirus 229E in L132 cells although they concluded that the drug is principally active during an early stage of the viral growth cycle. None of these experiments exclude the possibility that actinomycin D prevents the transcription of short-lived host mRNA essential for viral replication. Another possibility is that the transport of an essential RNA species from the nucleus to cytoplasmic sites of viral biosynthesis is blocked by the action of this drug (Levis and Penman, 1977).

An intriguing unanswered question concerns the precise role of host RNA polymerase II in the IBV replication cycle. Specific inhibition of this enzyme in IBV-infected BHK-21 cells by α -amanitin negates virus-stimulated [³H]uridine incorporation which is normally insensitive to the inhibitory effects of actinomycin D. It should be determined whether the appearance of all normal size classes of IBV-specific RNA is prevented in these cells by α -amanitin as a preliminary sign that the initiation and possible maintenance of viral RNA synthesis depend on the functional integrity of nuclear RNA polymerase II. For influenza virus, there is convincing evidence that this eucaryotic enzyme provides a host mRNA primer which serves to initiate viral RNA transcription in the cell nucleus (Mark *et al.*, 1979; Plotch *et al.*, 1979). RNA polymerase II as a multicomponent enzyme which exists in at least three structurally distinct forms has been directly implicated in the synthesis of viral mRNAs (or their precursors) of various DNA viruses and RNA retroviruses (Roeder, 1976). While the avian coronaviruses are credited with possessing the largest single-stranded RNA genome (Lomniczi and Kennedy, 1977), the manner in which it

is replicated is presently unknown (Robb and Bond, 1979). One possibility is that IBV and other positive-stranded coronaviruses utilize a virus-coded RNA-dependent RNA polymerase which is a hybrid enzyme containing one or more host components including specific subunits derived from nuclear RNA polymerase II. The precedent for such multicomponent viral enzymes of mixed origin is already well documented for procaryotic species (Losik and Pero, 1976).

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REFERENCES

- BABLANIAN, R., EGGERS, H. J., and TAMM, I. (1965). Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. *Virology* **26**, 100–113.
- BARRY, R. D. (1964). The effects of actinomycin D and ultraviolet irradiation on the production of fowl plague virus. *Virology* **24**, 563–569.
- CARTER, S. B. (1967). Effects of cytochalasins on mammalian cells. *Nature (London)* **213**, 261–264.
- CHAN, V. L., WHITMORE, G. F., and SIMINOVITCH, L. (1972). Mammalian cells with altered forms of RNA polymerase II. *Proc. Nat. Acad. Sci. USA* **69**, 3119–3123.
- FOLLETT, E. A., PRINGLE, C. R., and PENNINGTON, T. H. (1975). Virus development in enucleate cells: Echovirus, poliovirus, pseudorabies virus, reovirus, respiratory syncytial virus and Semliki forest virus. *J. Gen. Virol.* **26**, 183–196.
- GOLDMAN, N., PRESSER, I., and SREEVALSAN, T. (1977). California encephalitis virus: Some biological and biochemical properties. *Virology* **76**, 352–364.
- HRUBY, D. E., GUARINO, L. A., and KATES, J. R. (1979). Vaccinia virus replication. I. Requirement for the host-cell nucleus. *J. Virol.* **29**, 705–715.
- KELLY, D. C., AVERY, R. J., and DIMMOCK, N. J. (1974). Failure of an influenza virus to initiate infection in enucleate BHK cells. *J. Virol.* **13**, 1155–1161.
- KENNEDY, D. A., and JOHNSON-LUSSENBURG, C. M. (1979). Inhibition of coronavirus 229E replication by actinomycin D. *J. Virol.* **29**, 401–404.
- LAI, M. M. C., and STOHLMAN, S. A. (1978). RNA of mouse hepatitis virus. *J. Virol.* **26**, 236–242.
- LAMB, R. A., and CHOPPIN, P. W. (1977). Synthesis of influenza virus polypeptides in cells resistant to alpha-amanitin: Evidence for the involvement of cellular RNA polymerase II in virus replication. *J. Virol.* **23**, 816–819.
- LEVIS, R., and PENMAN, S. (1977). The metabolism of poly (A)⁺ and poly (A)⁻ hnRNA in cultured *Drosophila* cells studied with a rapid uridine pulse-chase. *Cell* **11**, 105–113.
- LOBBAN, P. E., SIMINOVITCH, L., and INGLES, C. J. (1976). The RNA polymerase II of an α -amanitin-resistant Chinese hamster ovary cell line. *Cell* **8**, 65–70.
- LOMNICZI, B., and KENNEDY, I. (1977). Genome of infectious brochitis virus. *J. Virol.* **24**, 99–107.
- LOSIK, R. M., and PERO, J. (1976). Regulatory subunits of RNA polymerase. In: "RNA Polymerase" (R. Losik and M. Chamberlin, eds.) pp. 227–246. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
- MAHY, B. W. J., HASTIE, N. D., and ARMSTRONG, S. J. (1972). Inhibition of influenza virus replication by α -amanitin: Mode of action. *Proc. Nat. Acad. Sci. USA* **69**, 1421–1424.
- MARK, G. E., TAYLOR, J. M., BRONI, B., and KRUG, R. M. (1979). Nuclear accumulation of influenza viral RNA transcripts and the effects of cycloheximide, actinomycin D and α -amanitin. *J. Virol.* **29**, 744–752.
- MCINTOSH, K. (1974). Coronaviruses: A comparative review. *Curr. Top. Microbiol. Immunol.* **63**, 85–129.
- OBJESKI, J. F., and MURPHY, F. A. (1977). Bunyaviridae: Recent biochemical developments. *J. Gen. Virol.* **37**, 1–14.
- OTSUKI, K., NORO, K., YAMAMOTO, H., and TSUBOKURA, M. (1979). Studies on avian infectious bronchitis virus (IBV). II. Propagation of IBV in several cultured cells. *Arch. Virol.* **60**, 115–122.
- PLOTCH, S. J., BOULOY, J. M., and KRUG, R. M. (1979). Transfer of 5' terminal cap of globin mRNA to influenza viral complementary RNA during transcription *in vitro*. *Proc. Nat. Acad. Sci. USA* **76**, 1618–1622.
- ROBB, J. A., and BOND, C. W. (1979). Coronaviridae. In "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), Vol. 14, Chap. 3, pp. 193–247. Plenum, New York.
- ROEDER, R. G. (1976). Eukaryotic nuclear RNA polymerases. In "RNA Polymerase" (R. Losik and N. Chamberlin, eds.), pp. 285–329. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
- ROTT, R., SABER, S., and SCHOLTISSEK, C. (1965). Effect on myxovirus of mytomycin C, actinomycin D, and pretreatment of the host cell with ultraviolet light. *Nature (London)* **205**, 1187–1190.
- SCHOCHETMAN, G., STEVENS, R. H., and SIMPSON,

- R. W. (1977). Presence of infectious polyadenylated RNA in the coronavirus avian bronchitis virus. *Virology* **77**, 772-782.
- SILVER, M., MCFADDEN, G., WILTON, S., and DALES, S. (1979). Biogenesis of poxviruses: Role for the DNA-dependent RNA polymerase II of the host during expression of late functions. *Proc. Nat. Acad. Sci. USA* **76**, 4122-4125.
- SPOONER, L. L., and BARRY, R. D. (1977). Participation of DNA-dependent RNA polymerase II in replication of influenza viruses. *Nature (London)* **268**, 650-652.
- TYRRELL, D. A., ALEXANDER, D. J., ALMEIDA, J. D., CUNNINGHAM, C. H., EASTERDAY, B. C., GARWES, D. J., HIERHOLZER, J. C., KAPIKIAN, A., MACNAUGHTON, M. R., and MCINTOSH, K. (1978). Coronaviridae: second report. *Intervirology* **10**, 321-328.
- WIKTOR, T. J., and KOPROWSKI, H. (1974). Rhabdovirus replication in enucleated host cells. *J. Virol.* **14**, 300-306.
- YAMADA, S. M., OCHIAI, H., FUJIKAWA, Y., UCHIFU, Y., and KODA, Y. (1971). Tissue culture and attenuation of avian infectious bronchitis virus. I. Tissue culture and passage of virus. *J. Japan. Vet. Med. Assoc.* **24**, 121-129.