PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 688-693 (1975)

Replication of IPN Virus: A Cytochemical and Biochemical Study in SWT Cells (38611)

RUSSELL K. KELLY AND PHILIP C. LOH

Virus Laboratory, Department of Microbiology, University of Hawaii, Honolulu, Hawaii 96822

Although the growth characteristics of infectious pancreatic necrosis (IPN) virus have been determined in a number of fish cell lines (1), little is known concerning its replication in the susceptible host cell. The virion is the causal agent of a high-mortality disease of trout fingerlings (1). Recent electron-microscopical and biochemical evidence indicate that the virion has an icosahedral structure of 74 nm diameter containing a single nonsegmented RNA component of molecular wt 3.1×10^6 (2). The viral RNA is single-stranded but has properties which suggest an unusual secondary structure (2). The present report examines the growth and macromolecular synthesis of IPN virus in an established cell line derived from the embryonic tissues of the red swordtail *Xiphophorus helleri* (3).

Materials and Methods. Virus. The IPN virus (ATCC VR 299) was kindly supplied by Kenneth E. Wolf of the Eastern Fish Disease Laboratory, Kearneysville, W. VA. Stock cultures of the virion were prepared in RTG-2 cells (rainbow trout gonads) at 22°. Virion infectivity titrations were done in tube cultures of RTG-2 cells and the 50% end point was calculated according to the method of Reed and Muench (4).

Cell culture. The swordtail cell line (SWT) was propagated as monolayers in Eagle's Basal Medium (EBM) plus 10% fetal calf serum (10 FCS) and maintained in the manner described previously (3). The RTG-2 cells were obtained from the American Type Culture Collection CCL 55 and were grown in EMB 10 FCS.

Virus growth. To determine the growth kinetics of IPN virus in SWT cells, cell monolayer cultures were infected with virion at an exposure multiplicity (10 $TCID_{50}$/cell) sufficient to yield a single cycle of growth of the virion. After an adsorption period of 2.5 to 3 hr at 22°, the cultures were washed twice with Hank's BSS, and experimental medium EBM2FCS was introduced. Sets of cultures were incubated at 16°, 22°, and 30° and at various intervals after infection, duplicate cultures at each temperature were removed, and the total yield of virion determined.

In certain experiments the replication of IPN virus in SWT cells in the presence of varying concentrations (0.01 μg/ml–1.0 μg/ml) of the antibiotic Actinomycin D (AD) was examined. Since AD selectively inhibits DNA-dependent RNA synthesis (5) and not RNA-dependent RNA synthesis, it can be used to distinguish the replication of RNA containing virions from that of DNA-containing virions. Furthermore, the antibiotic does not interfere with the replication of double-stranded RNA-containing virions such as the reoviruses.

Macromolecular synthesis and assay. The method employed to follow macromolecular synthesis in IPN-infected SWT cells, using the labeled precursors ^{3}H-thymidine and ^{3}H-uridine, was according to that previously described (6). Briefly, monolayers of SWT cells infected with IPN virus (10–20 $TCID_{50}$/cell) sufficient to yield a single cycle of growth were at various periods after infection pulse labeled for 2 hr with 3.3 μCi/ml of each tritiated nucleoside. After the labeling period the cell monolayers were removed by gentle scraping, washed three times with cold Dulbecco's phosphate-buffered saline (D-PBS) and acid precipitated in the cold with perchloric acid (PCA) (5%) for 30 min. The precipitates were then recovered, washed with 5% PCA on Millipore filters, and after air-drying and addition of Permafluor-toluene (Packard), the amount of radioactivity was determined in a liquid scintillation spectrometer. Uninfected monolayers of SWT cells served as controls.

Acridine orange staining. The acridine-orange (AO) fluorochrome staining technique described previously (7) was used to

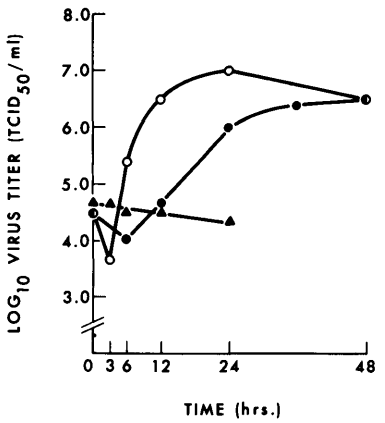

FIG. 1. Growth kinetics of infectious pancreatic necrosis virion in SWT cells at different temperatures. Total virion synthesized at 16° ●——●, 22° ○——○, and 30° ▲——▲. For each time period, total virion yields of IPN virion from infected SWT cells in duplicate 1 oz prescription bottles were pooled and assayed by $TCID_{50}$, as described in the text.

stain coverslip preparations of SWT and RTG-2 cells infected with IPN virus at a sufficient multiplicity (10 $TCID_{50}$/cell) to yield a single cycle of growth of the virion. At various intervals after infection duplicate coverslips were removed, washed with BSS and fixed in cold acetone (10 min). After air drying the coverslips were stained with AO and examined under a Zeiss fluorescent microscope. Uninfected cell preparations served as controls. In certain experiments the coverslips were pretreated with either bovine pancreatic ribonuclease (RNase, 100 μg/ml in distilled water), or deoxyribonuclease (DNase, 100 μg/ml in D-PBS) for 1 hr at 37°, before staining.

All of the experiments described below were performed at least twice and in some instances three to four times.

Results. Virus growth kinetics. The growth kinetics of IPN virus in SWT cells were examined at different temperatures (Fig. 1). At 22C the IPN virion replicated in SWT cells with an eclipse period of between 3 to 6 hr and the synthesis of virion was completed by 24 hr postinfection (p.i.), at which time maximum yields of 45–50 $TCID_{50}$ units per cell were attained. At 16° the synthesis of virion was slower with an eclipse period lasting nearly 12 hr and maximum virion titers were not attained until about 36 hr p.i. Although the IPN virus was found to be stable at 30° for at least 24 hr (unpublished observation), the virion failed to replicate at this temperature. Furthermore, the SWT cells have been found to grow readily at 30° but not at lower temperatures (16°) (3). In the present study the lower temperature supports growth of the virion.

Effect of actinomycin D. The antibiotic AD has been reported to selectively inhibit DNA- but not RNA-dependent RNA synthesis (5) (8) perhaps by binding specifically with deoxyguanosine residues and thereby directly blocking, or displacing, RNA polymerase (9) (10). Because of the above property AD has been used to distinguish between the replication of RNA-containing from that of DNA-containing virions (5). Within the former group is included the double-stranded RNA-containing virions of the reovirus (11) (12). However, certain RNA-containing virions, such as the leukoviruses and orthomyxoviruses, are inhibited by low concentrations of AD (13–17). The mechanism of inhibition in the case of the leukoviruses is apparently at a viral-induced DNA intermediate sequence (18) and in the case of the orthomyxoviruses an early specific cellular DNA-dependent function for replication (16, 19).

The replication of IPN virus was found to be inhibited by as little as 0.01 μg/ml of AD. At this concentration virion yields in SWT cells were markedly inhibited by greater than 50% (Table I). In contrast AD at a concentration of 0.1 μg/ml did not affect the synthesis of the double-stranded RNA-containing reovirus.

Synthesis of RNA and DNA. The effect of IPN infection on cellular DNA and RNA synthesis in SWT cells was followed by pulse label with either ^{3}H-thymidine or ^{3}H-uridine respectively, at various intervals after infection (see Materials and Methods). The infection of SWT cells at 22° resulted in an early inhibition of cellular DNA synthesis as indicated by a decreased incorporation of labeled thymidine into the acid-insoluble fraction of the infected cells by 2 hr p.i. (Fig. 2B). In contrast, total RNA synthesis appears elevated commencing within 2 hr p.i. and attaining a peak at 8 hr p.i., after

TABLE I. EFFECT OF ACTINOMYCIN D (AD) ON THE REPLICATION OF IPN VIRUS AND REOVIRUS TYPE 2.

Concentration of AD(μg/ml)[a]	IPN virus[b] Log. $TCID_{50}$/ml	% Reduction	Reovirus[c] IU/ml × 10^4	% Reduction
0	8.0	0	1042	—
0.01	7.7	50	ND[d]	—
0.05	7.5	68	ND	—
0.1	6.7	95	1286	0
1.0	6.7	95	8	100

[a] Pretreatment for 2 hr and posttreatment until virion is harvested.

[b] Residual virion after adsorption $\leq 10^{3.5}$ $TCID_{50}$/ml. Total virion yield after 42 hr p.i.

[c] Residual virion after adsorption was 10 × 10^4 infections units (IU)/ml. Total virion yield determined at 24 hr p.i. by the immunofluorescent assay-technique.

[d] ND = not done.

which time synthesis declined (Fig. 2A). This period of rapidly increasing RNA synthesis appears to coincide with the exponential production of infectious virus and may very likely represent viral RNA. Addition of AD at 0.05 μg/ml effectively suppressed the increased RNA activity.

Acridine orange staining. The fluorochrome dye acridine orange (A.O.) is a useful histochemical stain to selectively differentiate between single-stranded and double-stranded nucleic acids (20). Furthermore, the dye, when employed in combination with specific-nuclease treatment, has been useful in the differentiation of the type of nucleic acids present in the inclusion bodies found in certain virion-infected cells (7). In the present experiment SWT and RTG-2 cells infected with IPN virus were fixed and stained with A.O. at different intervals after infection (see Materials and Methods).

Examination of A.O. stained SWT cells and RTG-2 cells infected with IPN virus revealed the presence of metachromatically (red–orange) staining inclusion bodies in the cytoplasm of both kinds of cells (Fig. 3B). The inclusions were present in the majority of cells late in the infectious cycle (16–20 hr) and were digestible by pancreatic RNase (100 μg/ml in distilled water), but not by DNase (100 μg/ml in D-PBS). No

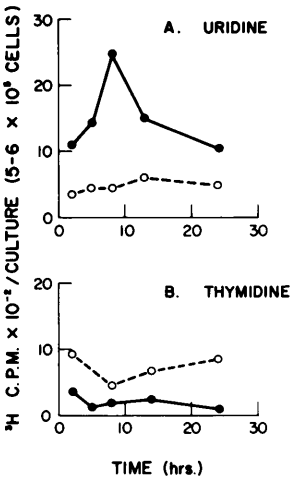

FIG. 2. Nucleic acid synthesis: A. Total RNA synthesis in SWT cells, uninfected O-----O and infected ●——● with infectious pancreatic necrosis virus. B. Total DNA synthesis in SWT cells, uninfected O-----O and infected ●——● with infectious pancreatic necrosis virus. The specific activity for each 2-hr pulse represents the average radioactivity of duplicate samples of incorporated labeled nucleoside (3.3 μCi/ml) into the acid insoluble fraction of 5–6 × 10^5 cells and is expressed as a function of time. The temperature for viral infection and replication was 22°.

orthochromatically green-staining cytoplasmic inclusion bodies, comparable to those described in cells infected with reoviruses were seen (21). Thus, the inclusion bodies of IPN-infected cells appear to be composed of single-stranded RNA.

When the time of appearance of the inclusion bodies was compared to RNA synthesis in the infected cell, the former structures were found well after maximum RNA synthesis had occurred. The role of the inclusion bodies in relation to production of infectious virion is not known.

Discussion. The present data indicate that the growth kinetics of IPN virus in SWT cells is essentially similar to that obtained in other fish cell systems such as, RTG-2 cells (22), BF-2 cells (23), and GF cells (24). Of significance is the observation that IPN virus is capable of replication in SWT cells at 16°, a temperature inhibitory to cellular growth. In contrast the virion did not replicate at 30°, a temperature which does not affect the viability of the cell. The

correlates rather well with the exponential production of infectious virion. In contrast, the synthesis of DNA is depressed. These results are in general agreement with the observations made for IPN virus infection of other cell systems (22) (25). However, in these studies conflicting results were obtained in regard to the nature of the newly synthesized RNA in IPN-infected cells. Argot (22) on the basis of A.O. staining of inclusion bodies in IPN-infected BF-2 cells and thermal denaturation studies of viral RNA extracted from the virion, interpreted the data as evidence for the double-stranded nature of the viral RNA. It should be noted that these inclusion bodies were infrequently observed and nuclease digestion data were not presented. In contrast, in a radioautographic study of IPN virus-infected RTG-2 cells, Nicholson (25) found that the newly synthesized RNA was sensitive to ribonuclease digestion and that only red–orange-staining cytoplasmic material was observed in AO-staining virion infected cells. On this basis he suggested that IPN–RNA was probably single-stranded. Our current observations are essentially in agreement with those of Nicholson's (25). At no time were green-staining cytoplasmic inclusion bodies, suggestive of double-stranded nucleic acid structures, observed in infected cells. Furthermore, our recent biochemical studies indicate that the viral RNA from highly purified IPN virus is a single-stranded nonsegmented component of molecular wt 3.1×10^6 daltons and possessing unusual secondary structure (26). However, since a recent report has provided some evidence to suggest that the IPN genome may possibly be a double-stranded RNA structure (27), the question of the exact structure of its viral RNA remains still to be definitively resolved.

The significance of the numerous red–orange cytoplasmic structures, sensitive to ribonuclease digestion, and seen late in the viral replication cycle, after maximum RNA synthesis had occurred, remains to be determined. Basophilic cytoplasmic inclusion bodies have been observed in histological preparations of pancreatic acinar cells of IPN-infected trout (28) and also in primary trout cells infected with the virion (29, 30). Similarly, cytoplasmic inclusions have been observed in electron micrographs of thin-sectioned preparations of pancreatic tissue of naturally infected trout (31). Here, many of these structures were found to be essentially comprised of either crystalline aggregates of the virion (31) or consisted of cell debris plus virion (31). The relationship of the A.O.-staining cytoplasmic inclusion bodies observed in these studies to the structures observed under the electron microscope is unclear and remains to be resolved.

The growth of IPN virus in RTG-2 cells was previously reported to be inhibited by 2 μg/ml of AD (22, 23). In contrast the present data indicate that as little as 0.05 μg/ml of AD markedly inhibited virion yields in SWT cells ($>50\%$). Also, Nicholson (32) has reported that a concentration of 0.2 μg/ml of the antibiotic effectively inhibited virion production in RTG-2 cells by 99%. This sensitivity of the IPN virus to AD differs from that of the double-stranded RNA-containing reovirus (11) (12). The pattern of inhibition appears to parallel in many ways that described for the orthomyxoviruses and poliovirus (16, 33, 34). Although the mechanism of inhibition of IPN virion replication by AD is not known, the AD sensitive step in the replication of IPN virus occurs early in the infectious sequence (32).

Summary. Although IPN virus failed to multiply at 30°, it replicated at 16° and 22° in SWT cells. At 22° the viral eclipse period lasted nearly 6 hr with maximal virion titers attained by 24 hr, whereas replication at 16° was much slower. The replication of the virion was inhibited by 0.05 μg/ml of AD which did not interfere with the production of reovirus. Biochemical studies revealed that cellular DNA synthesis was markedly reduced ($>50\%$) soon after infection, whereas total RNA synthesis was enhanced. The period of rapid increase in RNA synthesis paralleled the exponential production of infectious virus. Viral inclusion bodies, revealed by acridine orange-staining of virus-infected cells (SWT and RGG-2) late in the infectious cycle, were found to contain single-stranded RNA on the basis of their staining characteristics and sensitivity to RNase.

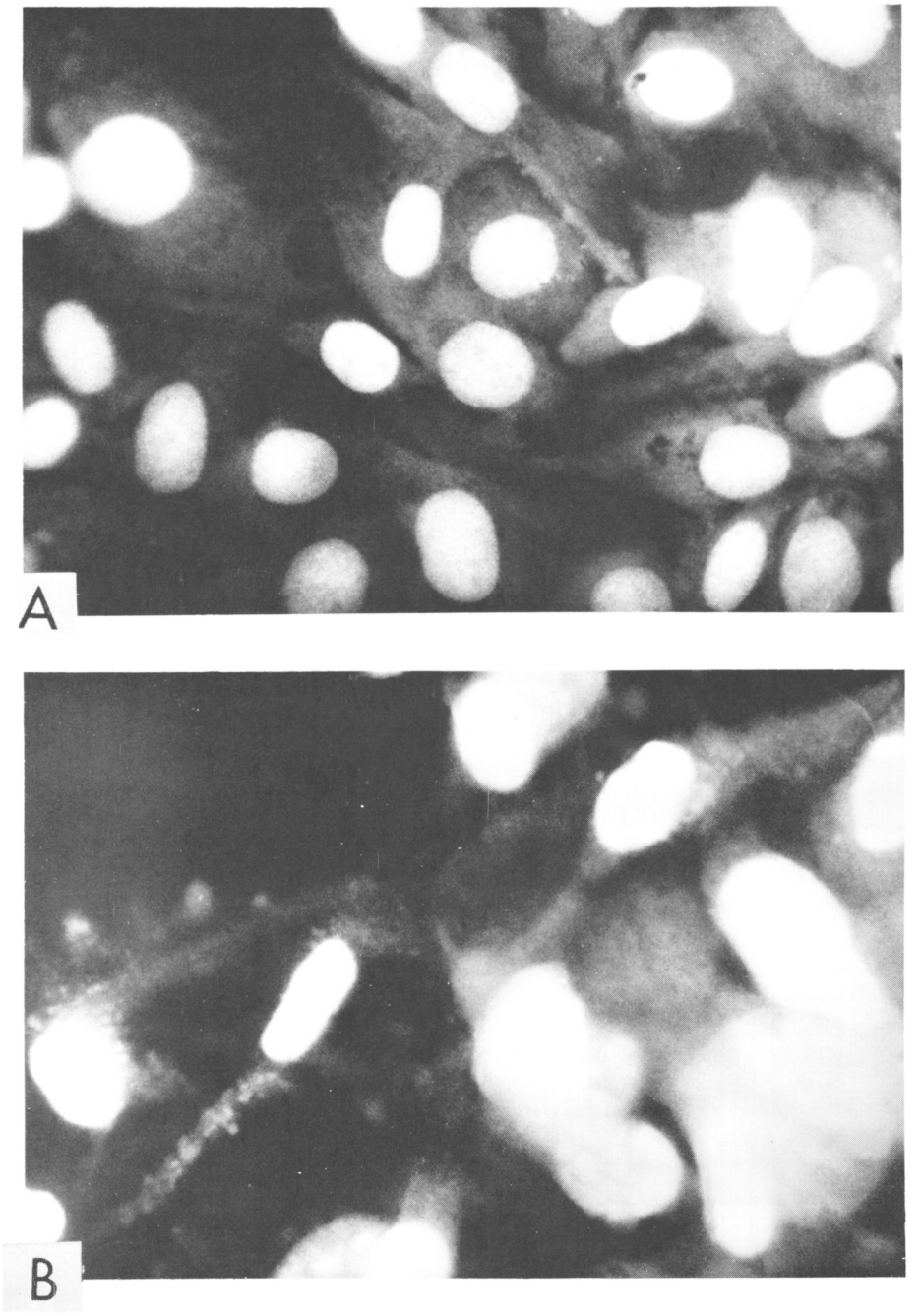

FIG. 3. Acridine-orange staining of RTG-2 cells infected with infectious pancreatic necrosis virus: A. Uninfected RTG-2 cells (× 1540). B. IPN virus-infected RTG-2 cells 16 hr after infection (× 1540).

inhibitory effect of higher temperature (33°) on IPN virus replication in FHM cells has been described (23). A plausible explanation for this phenomenon is that the viral mediated enzymes necessary for IPN virus replication possess optimal temperature requirements which differ from those of either of these cell systems.

The maximum levels of RNA synthesis in IPN-infected SWT cells (6–8 hr p.i.) at 22°,

1. Wolf, K., Advan. Virus Res. **12,** 35 (1966).
2. Kelly, R. K., and Loh, P. C., J. Virol. **10,** 824 (1972).
3. Kelly, R. K., and Loh, P. C., *In Vitro* **9,** 73 (1973).
4. Reed, L. J., and Meunch, H., Amer. J. Hyg. **27,** 493 (1938).
5. Reich, E., Franklin, R. M., Shatkin, A. S., and Tatum, E. L., Science **134,** 556 (1961).
6. Loh, P. C., and Oie, H. K., J. Virol. **4,** 890 (1969).
7. Armstrong, J. A., and Niven, J. S. F., Nature (London) **189,** 1335 (1957).
8. Goldberg, I. H., and Rabinowitz, Science **136,** 315 (1962).
9. Hamilton, L., Fuller, W., and Reich, E., Nature (London) **198,** 538 (1963).
10. Goldberg, I. H., and Rabinowitz, Science **136,** 315 (1962).
11. Shatkin, A. J., Biochem. Biophys. Res. Commun. **19,** 506 (1965).
12. Loh, P. C., and Soergel, M., Proc. Soc. Exp. Biol. Med. **122,** 1248 (1966).
13. Temin, H. M., Virology **20,** 577 (1963).
14. Allen, D. W., Biochim. Biophys. Acta. **114,** 606 (1966).
15. Duesberg, P. H., and Robinson, S. W., Virology **31,** 742 (1967).
16. Barry, R. D., *in* "Effect of Inhibitors of Nucleic Acid Synthesis on the Production of Myxoviruses" (G. E. Wolstenholme and J. Knight, eds.), p. 51, Little and Brown, Boston (1964).
17. Granoff, A., and Kingsbury, D. W., *in* "Effect of Actinomycin D on the Replication of Newcastle Disease and Influenza Virus" (G. Wolstenholme and J. Knight, eds.) p. 120, Little and Brown, Boston (1964).
18. Temin, H. M., Virology **23,** 486 (1964).
19. Borland, R., and Mahy, B. W. J., J. Virol. **2,** 33 (1967).
20. Spendlove, R. S., *in* "Microscopic Techniques" (K. Maramorosch and H. Koprowski, eds.), p. 425, Methods in Virology III, Academic Press, New York (1967).
21. Oie, H. K., Loh, P. C., and Soergel, M., Arch. Gesamte Virusforsch **18,** 16 (1966).
22. Argot, J. E., "Infectious Pancreatic Necrosis Virus Intracellular Replication", Doctoral Dissertation, Lehigh University, 102 pp. Microfilms, Ann Arbor (1969).
23. Gravell, M., and Malsberger, R. G., Ann. N. Y. Acad. Sci. U.S.A. **126,** 555 (1965).
24. Moewus-Kobb, L., Ann. N. Y. Acad. Sci. U.S.A. **126,** 328 (1965).
25. Nicholson, B. L., J. Gen. Virol. **13,** 369 (1971).
26. Kelly, R. K., and Loh, P. C., J. Virol. **10,** 824 (1972).
27. Cohen, J., Poinsard, A., and Scherrer, R., J. Gen. Virol. **21,** 485 (1973).
28. Wood, E. M., Snieszko, S. F., and Yasutake, W. T.,A.M.A. Arch. Pathol. **60,** 26 (1955).
29. Wolf, K., Quimby, M. C., Pyle, E. A., and Dexter, R. P., Science **132,** 1890 (1960).
30. Parisot, T. J., Yasutake, W. T., and Bressler, V., Trans. Amer. Fisheries Soc. **92,** 63 (1963).
31. Lightner, D., and Post, G., J. Fish. Res. Board Can. **26,** 2242 (1969).
32. Nicholson, B. L., Experientia **27,** 1362 (1966).
33. Cooper, P. D., Virology **28,** 663 (1966).
34. Schaffer, F. L., and Gordon, M., J. Bacteriol. **91,** 2309 (1966).

Received September 6, 1974. P.S.E.B.M. 1975, Vol. 148.