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Restricted Replication of Frog Virus 3 in Selected Variants of BHK Cells

R. VILAGINÈS[1] AND B. R. MCAUSLAN
Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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Synthesis and maturation of frog virus 3 deoxyribonucleic acid (DNA) in BHK cells and selected variants, in chick fibroblasts, and in minnow cells were compared. Wide ranges in rates of DNA synthesis and assembly of virions were found. At least three variants of BHK cells can be obtained: (i) fully permissive, characterized by rapid DNA synthesis and assembly to give a high yield of infective virus; (ii) semipermissive, in which viral DNA is synthesized slowly for extended periods of time, and the yield of infective virus is poor; and (iii) nonpermissive, in which virus adsorbs to cells and arrests host functions but viral DNA is not synthesized. Kinetics of appearance of virions and procedures for their separation from cell extracts are described.

Frog virus 3 (FV_3), a polyhedral deoxyvirus of amphibia, will replicate in the cytoplasm of mammalian cells (5, 6). Cell systems in which this virus will replicate, and techniques for its isolation and assay, have been developed largely through the efforts of Granoff and co-workers (1, 2). FV_3 deoxyribonucleic acid (DNA) has a molecular weight and guanine-cytosine content estimated to be 130×10^6 daltons and 56%, respectively (9).

Using a line of baby hamster kidney cells (BHK) selected from BHK 21/13 (9) for their characteristics in suspension, Smith and McAuslan made the following observations for FV_3 infected cells: FV_3 rapidly arrested host DNA synthesis, viral DNA synthesis commenced 3 hr postinfection, and increase in infective virus was first detectable between 4 and 6 hr. The infection cycle terminated between 12 and 16 hr postinfection. Viral DNA was synthesized in excess of that packaged into virions; "free" viral DNA associated tenaciously with the nuclear fraction of disrupted cells, but "packaged" DNA remained in the cytoplasmic fraction. This type of cell-virus interaction we shall refer to as a fully permissive or "fast" system.

Consideration of other reports on the synthesis of FV_3 in BHK cells revealed that viral DNA synthesis continued for at least 48 hr post-infection and that viral DNA synthesis sometimes proceeded well beyond the time that formation of infective particles ceased (4).

It occurred to us that such studies may have been conducted in variants of the original BHK 21/13 cell, selected inadvertently by repeated subculture. We applied mild selection pressures to our line of BHK cells and have obtained variants in which FV_3 DNA synthesis and assembly are markedly different from the "fast" system. Such variants are potentially useful for studying the control of viral DNA synthesis and maturation. Studies with the variants are compared with results with other cell systems.

[1] Roche postdoctoral fellow on leave from the Pasteur Institute, Paris, France.

MATERIALS AND METHODS

Virus. FV_3 was propagated and titrated in minnow cells (FHM; 6).

Infection. Cells in suspension were infected by methods described elsewhere (5). Unless otherwise stated, an input multiplicity of 10 plaque-forming units (PFU) per cell was used. To infect monolayer cultures, medium was removed, and virus (10 to 20 PFU/cell) in 0.2 ml was added and allowed to adsorb at room temperature for 60 min before readdition of medium.

BHK cells. A line of BHK 21/13 cells (10) designated BHK-S (5) was used at a passage number less than 20 as the standard permissive system. This cell line was deliberately subcultured for 100 passages as a heavy confluent monolayer. It was then cloned, and a culture of one such clone in its tenth passage was designated BHK-Slo. From heavy confluent monolayers of BHK-S cells in their 100th passage, loosely attached cells were gently shaken free; these detached cells were subcultured for 10 passages and were designated BHK-Snon. For experiments, such cells were placed in suspension (5) and used the first or second day thereafter. These cells supported the replication of rabbitpox as efficiently as did BHK-S cells (McAuslan and Vilaginès, *in press*).

Other cells. A permanent line of minnow cells, FHM

(3), was used. Chick embryo fibroblasts were prepared as secondary monolayers.

Medium. Eagle's medium (Hanks base) supplemented with 10% heat-denatured fetal calf serum and 10% Tryptose-phosphate broth was used for monolayer cultures of BHK cells and FHM cells. Eagle's modified medium was used in all suspension systems. For growth and maintenance of chick fibroblasts, we used medium 199 supplemented with 10% Tryptose-phosphate broth and 2% heat-denatured fetal calf serum.

Susceptibility of cytoplasmic DNA to deoxyribonuclease. Cytoplasmic extracts of infected cells were incubated with 100 μg of deoxyribonuclease (Sigma Chemical Co. pancreatic deoxyribonuclease I, once crystallized) per ml for 120 min at 37 C, and the acid-precipitable count was then determined.

RESULTS

DNA synthesis in fully permissive "fast" systems. Kinetics of FV_3 DNA synthesis in suspension cultures of BHK-S are presented in Fig. 1. These results are similar to those described before (5). Attention is directed to the following aspects, which have been discussed previously (5). Viral (cytoplasmic) DNA synthesis commenced about 3 hr postinfection and ceased about 12 to 15 hr postinfection. Upon disruption of cells, a large fraction of viral DNA associated with the nuclear fraction but was susceptible to exogenous deoxyribonuclease; DNA in the cytoplasmic fraction was largely deoxyribonuclease-resistant.

Separation of virions from infected BHK-S. BHK-S cells were infected with FV_3. Tritiated thymidine (specific activity, 4 Ci/mmole; 1 μCi/ml of culture) was introduced 1 hr postinfection. Cells were harvested 9 hr later, and a crude cytoplasmic fraction, prepared by disruption of cells in hypotonic buffer (8), was layered on a sucrose gradient (20 to 70%, w/w) and centrifuged.

A photograph (Fig. 2a) of the resultant separation of components can be compared with the profile of acid-precipitable radioactivity in the gradient (Fig. 2b). Visible bands I, II, and III contained infective virus in the ratio of I + II:III = 10:1. Bands I and II formed only after infection, were difficult to separate, and sedimented very closely together as discrete bands on prolonged centrifugation. It was difficult to decide whether one or both bands contained thymidine. Therefore, fractions containing bands I and II were collected after prolonged centrifugation, as described in Fig. 2c, and were pooled. This material was recentrifuged in a CsCl gradient in an angle-head rotor. Two visible bands were again detected; only one sharp band of radioactivity was found, and the density of this band was 1.275 g/cc (Fig. 3). Values of 1.265 and 1.285 g/cc for FV_3 were reported by Smith and McAuslan (9) and by Morris et al. (7), respectively. The detailed composition and significance of band I is currently under investigation.

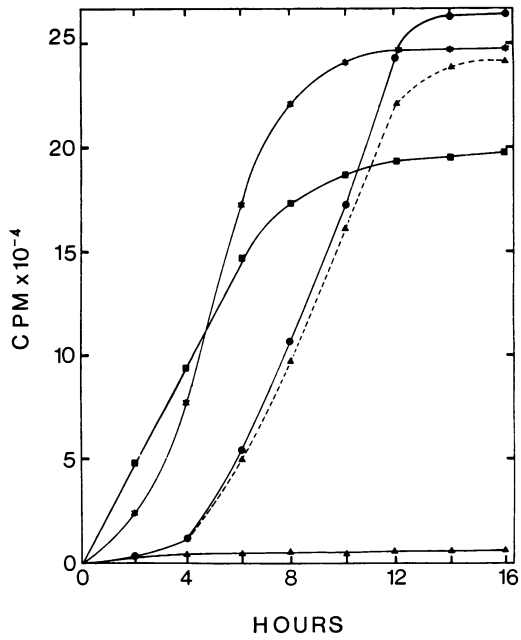

FIG. 1. *FV_3 DNA synthesis in BHK-S. Cells infected with FV_3 in suspension were incubated continuously at 29 C with ^3H-thymidine (1 μCi/ml; specific activity, 4 Ci/mmole). Samples of 5 × 10^6 cells were fractionated, and the incorporation of thymidine into acid-insoluble form was determined. Points represent incorporation into uninfected cell nuclei (■), infected nuclei (✱), uninfected cell cytoplasm (▲), infected cell cytoplasm (●), infected cell cytoplasm after nuclease treatment (▲--▲).*

The data of McAuslan and Smith (5) are incomplete in that infectivity was mentioned only for material banding at the position corresponding to band III (Fig. 2a). This represents virus adhering to cell material, and the amount of such material and the proportion of total virus adhering to it varied depending on the cells used.

FV_3 DNA synthesis in BHK variants. Experiments conducted as described for BHK-S cells were repeated with BHK-Slo (slow system). Host DNA synthesis was arrested after infection as effectively as it was in BHK-S, but, in marked contrast to the results obtained with the "fast" system, DNA synthesis in the infected cell cytoplasm proceeded slowly for 48 hr or more (Fig. 4). Up to 12 hr postinfection, 50 to 75% of this DNA was susceptible to exogenous deoxyribonuclease; after 12 hr, this susceptibility decreased. Coinciding with this was a progressive increase in thymidine-containing particles with a buoyant density

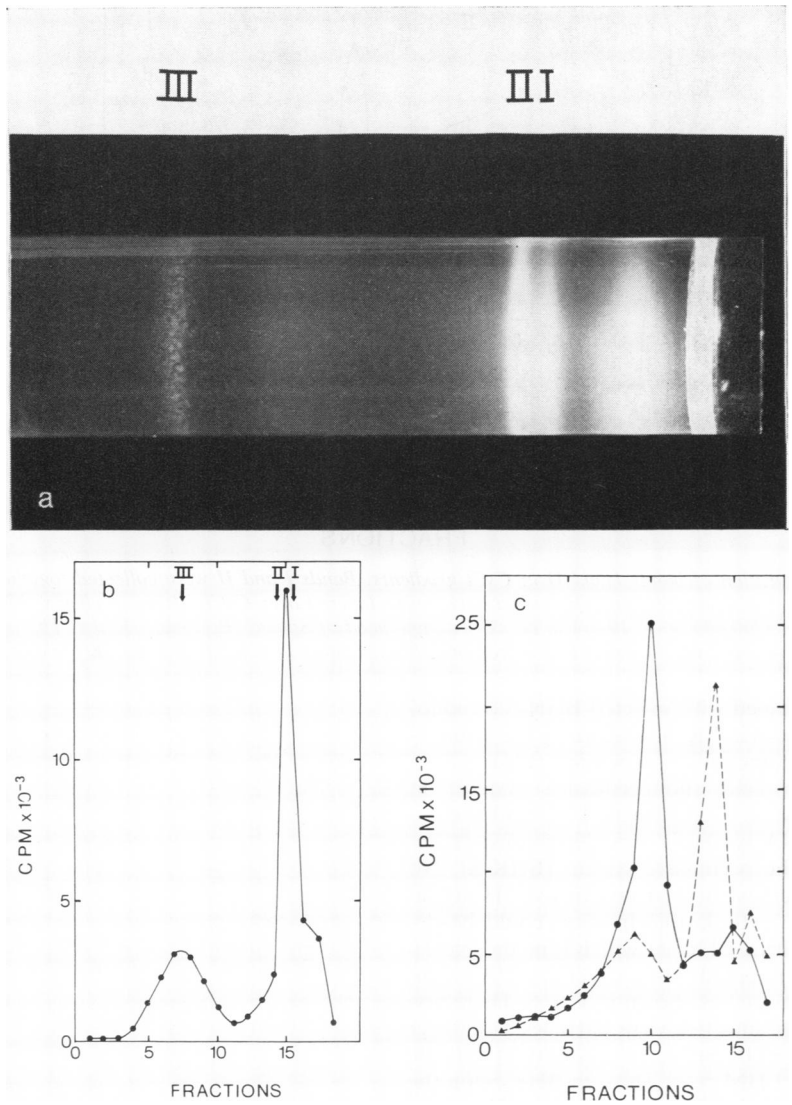

FIG. 2a. *Photograph of visible material separated on sucrose gradients. Cytoplasmic extract from BHK-S infected for 19 hr with FV$_3$ was layered on a 20 to 70% (w/w) sucrose gradient and centrifuged (30,000 × g, 20 min).*

FIG. 2b and c. *Sedimentation of ^{3}H-thymidine-labeled particles of infected BHK-S cells. (b) Cytoplasmic extracts of infected cells were layered over 16-ml sucrose gradients [20 to 70% (w/w) in reticulocyte-swelling buffer] and centrifuged (30,000 × g, 20 min). Visible bands I, II, and III correspond to those shown in Fig. 2a. Gradients were fractionated, and acid-insoluble radioactivity was determined. (c) Velocity sediment was conducted as described under Fig. 2a, but the position of ^{3}H-thymidine-labeled particles was determined in replicate gradients after centrifugation at 30,000 × g for 20 min (▲) or 180 min (●). In each case two discrete visible bands corresponding to bands I and II of Fig. 2a moved closely together over the position of the peak of ^{3}H activity.*

of 1.275 g/cc in CsCl, which sedimented in sucrose gradients (see Fig. 5) like those thymidine-containing particles (band II, Fig. 2a, 2b) produced in the fast system. At 48 hr postinfection, the yield of infective virus from BHK-Slo cells was only 3 PFU/cell. A small percentage of deoxyribonuclease-resistant radioactive material sedimented at the position found for band III with the fast system; however, no visible band coincided with this. Typical results for kinetics of viral DNA

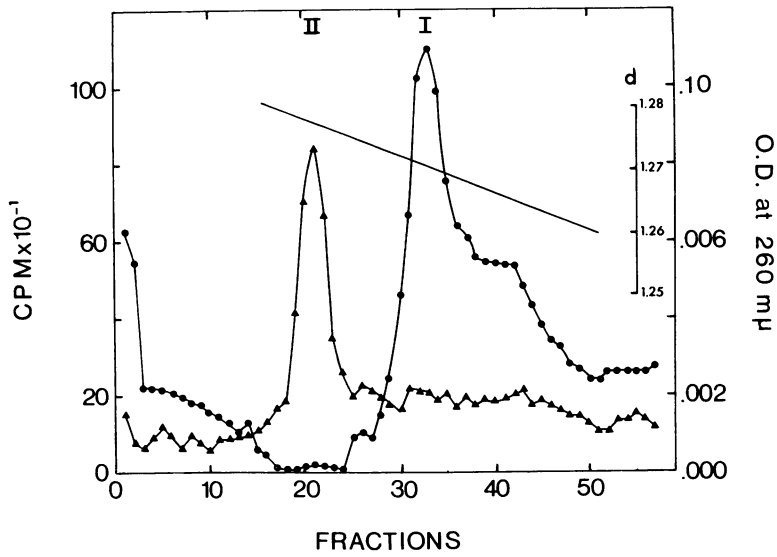

FIG. 3. *Separation of bands I and II in CsCl gradients. Bands I and II were collected together from sucrose gradients described under Fig. 2c. The mixed bands were layered over CsCl (10-ml gradients, 25 to 30% CsCl) and centrifuged (200,000 × g, 19 hr) in the 65 Ti angle head of Spinco (Beckman) Rotor. The two visible bands were clearly separated, and their positions corresponded to the major peak of* ^3H *activity* (▲) *or optical density* (●).

synthesis obtained with infected BHK-Slo monolayers are shown in Fig. 4. Essentially the same results were obtained with suspension cultures.

BHK-Snon cells (nonpermissive system) in suspension were infected with FV_3, and the kinetics of DNA synthesis were determined as described for the permissive system (BHK-S). No significant increase in incorporation of ^{3}H-thymidine into the cytoplasmic fraction could be detected for at least 24 hr, at which time the experiment was terminated. Host (nuclear) DNA synthesis was arrested as shown in Fig. 6. There was no synthesis of infective virus particles 24 hr after infection of BHK-Snon, as assayed by plaque formation in FHM cells. Thus, in BHK-Snon the virus that was adsorbed arrested host DNA synthesis but failed to initiate viral DNA synthesis.

DNA synthesis in other cells. Minnow cells (FHM) and chick embryo fibroblasts have been used to prepare virus stocks. We were interested in comparing the rates of DNA synthesis and appearance of virions in these cell lines to see whether they offered any advantage over BHK-S cells for production of virus.

FHM monolayers have been used for titration of virus by plaque assay and for production of stock virus (6). FHM cells were maintained in suspension in Eagle's medium (modified for suspension culture) supplemented with 5% fetal calf serum. These cells doubled approximately every 30 to 36 hr for at least 7 days. Kinetics of FV_3 DNA synthesis in such cells, 24 hr after they were

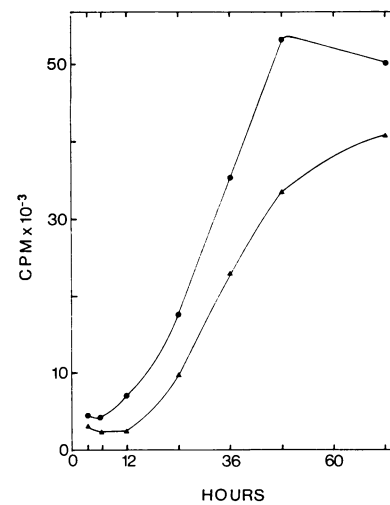

FIG. 4. *FV_3 DNA synthesis in BHK-Slo monolayers. Points represent incorporation of thymidine into cytoplasmic fraction of FV_3-infected BHK-Slo monolayers* (●) *and the fraction resistant to exogenous deoxyribonuclease* (▲). *Monolayers of 5 × 10^6 cells were labeled continously with ^3H-thymidine (10 μCi/ml; specific activity, 6.7 Ci/mmole).*

first placed into suspension, were determined as for the BHK experiments. Host DNA synthesis was arrested 2 hr postinfection. Viral DNA synthesis commenced 3 hr postinfection and proceeded for at least another 8 hr (Fig. 7). Most of

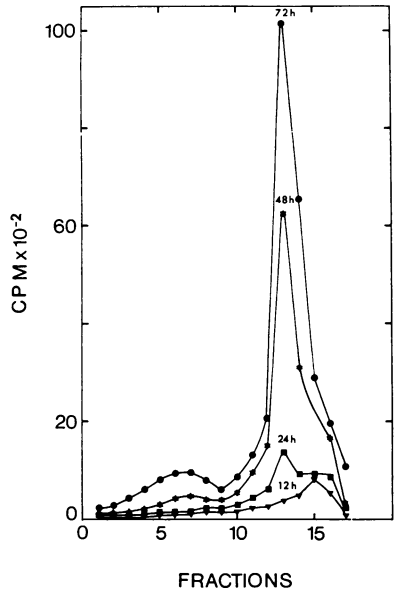

FIG. 5. *Kinetics of the appearance of ^{3}H-thymidine-labeled particles in FV$_3$-infected BHK-Slo. Cytoplasmic extracts prepared at intervals in the same experiment as described by Fig. 4 were centrifuged on sucrose gradients as described under Fig. 2b, and the increase in ^{3}H-thymidine-containing particles was determined after fractionating the gradients. Extracts prepared at 12 hr (▼), 24 hr (■), 48 hr (✱), and 72 hr (●) post-infection.*

the newly synthesized DNA remained in the cytoplasmic fraction after disruption of cells, and 85% of this DNA was susceptible to exogenous deoxyribonuclease. At 24 hr postinfection, the yield of infective virus was only 5 PFU per cell.

Confluent secondary chick embryo fibroblast monolayers were infected with FV$_3$. Tritiated thymidine (specific activity, 6.7 Ci/mmole; 10 μCi/ml) was added to each culture at the start of infection. At intervals postinfection, medium was aspirated from cultures; the cells were rinsed once with tris(hydroxymethyl)aminomethane-hydrochloride (0.1 M, *p*H 7.8, containing 0.001 M thymidine), scraped into 3 ml of hypotonic reticulocyte-swelling buffer, and disrupted with a Dounce homogenizer (8). Nuclei were removed by centrifugation (500 × *g*, 5 min), and the tritiated thymidine incorporated into acid-precipitable form in the cytoplasmic fraction was determined. Kinetics of thymidine incorporation and susceptibility of viral DNA to exogenous deoxyribonuclease are shown in Fig. 8. The results of such experiments varied markedly from one experiment to another, even when one stock of virus was used under identical conditions. Fig. 8A and 8B represent extremes of the variations encountered.

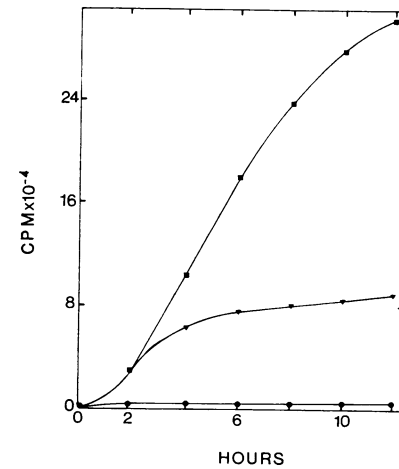

FIG. 6. *FV$_3$ DNA synthesis in BHK-Snon. BHK-Snon cells in suspension were infected and labeled as described for Fig. 1. After fractionation of cells at intervals, incorporation of ^{3}H-thymidine into acid-insoluble form in uninfected nuclear fraction (■), infected nuclear fraction (▼), and infected or uninfected cytoplasmic fraction (●) was determined.*

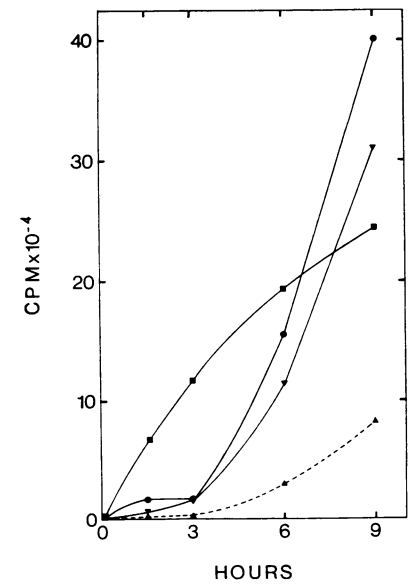

FIG. 7. *FV$_3$ DNA synthesis in FHM cells. FHM cells in suspension were infected with FV$_3$ and labeled continuously at 29 C with ^{3}H-thymidine (1 μCi/ml; specific activity, 4 Ci/mmole). At intervals, 10^7 cells were harvested, washed, and disrupted in reticulocyte-swelling buffer. Incorporation of ^{3}H-thymidine was determined on uninfected nuclear fraction (■), infected nuclear fraction (●), and infected cytoplasmic fraction (▼). Deoxyribonuclease-resistant radioactivity in the infected cytoplasmic fraction (▲--▲).*

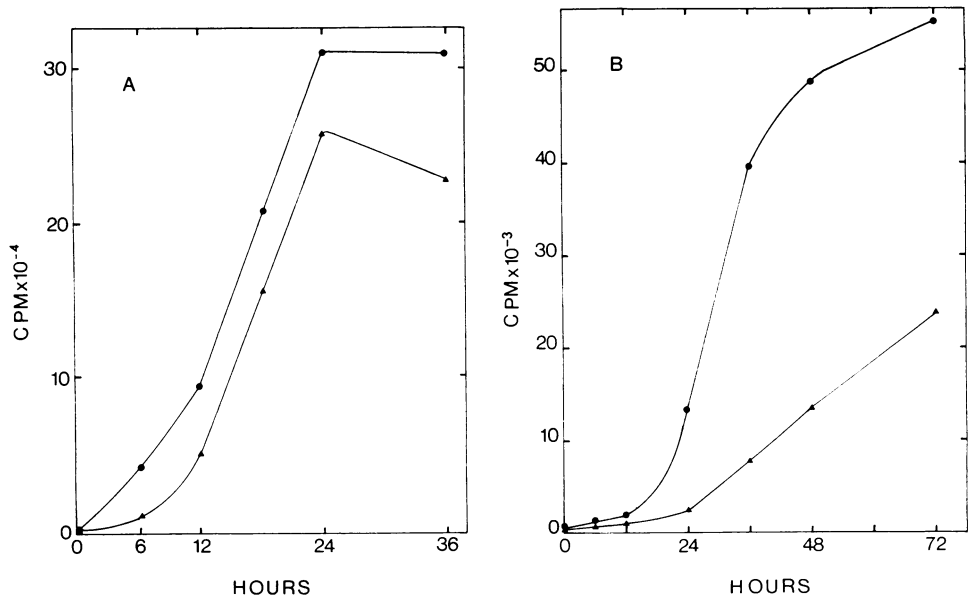

FIG. 8. *FV_3 DNA synthesis in chick embryo fibroblast cells. Chick embryo fibroblast monolayer cultures (5 × 10^6 cells, 60-mm dish) were infected with FV_3, at an input multiplicity of 10 PFU/cell. ^{3}H-thymidine was added after adsorption of virus, and cells were labeled continuously at 29 C. A and B represent extremes of variations encountered. Infected cytoplasmic fractions (●); deoxyribonuclease-resistant radioactivity in the infected cytoplasmic fraction (▲).*

Cytoplasmic extracts from infected cells in the same experiment described in Fig. 8B were analyzed for virus particles by velocity sedimentation through sucrose gradients. A progressive increase in thymidine-labeled particles occurred (Fig. 9); these particles had a buoyant density in CsCl of 1.275 g/cc. The yield of infective virus reach a maximum of 3 PFU per cell by 72 hr postinfection.

DISCUSSION

The data presented show that FV_3 was readily separated from cell extracts by zonal centrifugation in sucrose gradients, and only one type of deoxyribonuclease-resistant particles was found. Such particles had a density in CsCl (1.275 g/cc) similar to that reported previously for FV_3 particles [cf. Smith and McAuslan (9) and Morris et al. (7)], and this material banded in sucrose where infectivity banded.

There are few reports describing modification of an animal virus replication cycle resulting from variation in cells of the one host type. From the data presented here, it seems that, so far, FV_3 is known to replicate efficiently in only one cell system; thus, only this system would be suitable for a study of the biochemistry of FV_3 replication. On the other hand, restrictive cells should be useful for examination of some particular aspects of

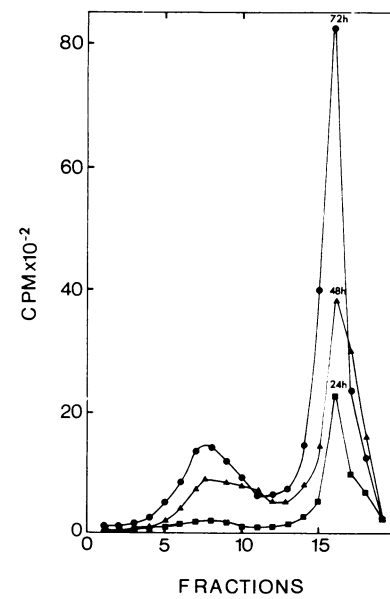

FIG. 9. *Kinetics of the appearance of ^{3}H-thymidine-labeled particles in FV_3-infected chick embryo fibroblasts. Cytoplasmic extracts prepared at intervals from infected cells in the same experiment as described by Fig. 8b were centrifuged on sucrose gradients as described under Fig. 2b, and the increase in ^{3}H-thymidine-containing particles was determined after fractionating the gradients. Extracts prepared 72 hr (●), 48 hr (▲), and 24 hr (■) postinfection.*

FV_3 macromolecular synthesis. Thus far, compared with the permissive system, we can show three situations: (i) conditions (BHK-Slo, chick embryo fibroblasts) where the rate of FV_3 DNA synthesis is slow and continues for prolonged periods up to six times longer than that in the fast system, and where detectable assembly of virus particles lags about 12 hr behind the initiation of DNA synthesis; (ii) cell types (FHM suspension, chick embryo fibroblast monolayers) in which DNA synthesis proceeds at a fast rate, but the DNA stays deoxyribonuclease-susceptible for a long period because assembly of particles is slow and inefficient; and (iii) cells in which viral DNA synthesis is negligible. We suggest that in future studies on the synthesis of FV_3 macromolecules it should be stated what particular situation exists relative to some standard, such as the fully permissive system described above.

We found in all systems that particles continued to be produced beyond the time that FV_3 DNA synthesis was arrested. On the other hand, Kucera and Granoff (4) reported that production of virions terminated well before termination of DNA synthesis. BHK cells produce many variants spontaneously, and with passage cell cultures may well have characteristics that are significantly different, in terms of supporting FV_3 replication, from the original BHK 21/13 clone. This situation may provide an answer for enquiries we have received from other investigators describing difficulties in getting FV_3 to replicate in any of the cell types used in published studies.

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