Nucleic Acid Synthesis in Cytoplasm of Yaba Monkey Tumor Virus-Infected Cells

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Yaba tumor virus progeny appeared in cynomolgus monkey kidney cells at 24 h postinfection and reached a plateau at 72 h in the first cycle of replication. Viral DNA synthesis was first detected at about 3 h and reached a peak in 18 h. Maximum coating of viral DNA in infected cells occurred at 4 days postinfection. Rapidly labeled RNA was synthesized in the cytoplasm of virus-infected cells. At 6 h postinfection 7 to 10S RNA was present; this species was present in greater amount at 12 h; at 24 h a truncated peak indicated the presence of 14 to 15S as well as 7 to 10S RNA. Hybridization data indicated that the largest peak of messenger RNA synthesis occurred at 11 to 13 h postinfection and a second, slightly smaller, peak occurred at 21 to 23 h.

Yaba virus replicates in primary rhesus monkey kidney (1), continuous cercopithecus kidney (22, 23), and continuous cynomolgus kidney (19, 20) cells. Nucleic acid synthesis in the cytoplasm of virus-infected cells has been studied by fluorescent and histochemical methods (15, 22) and by autoradiography (24). Yohn et al. (24) detected DNA synthesis at 3 h postinfection (PI), which reached a peak at approximately 20 h in cercopithecus monkey kidney cells. Kato et al. (15) described B-type inclusions characteristic of poxvirus-infected cells in the cytoplasm and showed that nuclear DNA synthesis was suppressed in cells bearing these inclusions as indicated by histological studies. Enhanced RNA synthesis was shown to occur in the cytoplasm at 36 h PI as determined by acridine orange and methyl green thionin staining (22).

In this investigation the synthesis of DNA in Yaba virus-infected cells was monitored by incorporation of acid-insoluble radioactive thymidine into the cytoplasmic fraction of established cynomolgus monkey kidney cells. The coating of viral DNA was indicated by study of the DNase sensitivity of labeled DNA contained in the cytoplasm of infected cells. The time course of virus-specific RNA was measured by hybridization with viral DNA and the size of the various species of viral RNA was estimated by density gradient centrifugation.

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MATERIALS AND METHODS

Cells. A continuous line of cynomolgus monkey kidney cells, Jinet (18), was used to propagate Yaba virus. Cells were grown in Eagle minimal essential medium (MEM) containing 10% fetal bovine serum (FBS).

Virus. Yaba virus was supplied by D. S. Yohn, Ohio State University. Virus was propagated in roller bottles (20 by 4 in.) (Bellco) and titered by plaque assay (20). Monolayers in roller bottles were infected with 0.5 to 1.0 PFU/cell in the presence of 0.01 mM MgCl₂ and allowed to adsorb for 2 h at 37 C. MEM containing 2% FBS was added and cells were incubated at 35 C for 6 days. Virus yield ranged from 10 to 13 OD₂₆₀ units of virus— 1.2×10^{10} viral particles per OD₂₆₀, (25)—per 8×10^7 cells but infectivity titer indicated that only about 1 in 500 viral particles was capable of plaque initiation.

Purification. The method for purification of poxviruses described by Joklik (11) was used to purify the virus. Electron microscopy revealed the purified preparations to contain about 85% uniform viral particles.

Single-step growth curve. Titration of Yaba virus during the first 96 h PI was made by the plaque method devised for Yaba virus by using 50 mM MgCl₂ in a second agar overlay to selectively kill infected cells (20).

Incorporation of radioactive thymidine. Titration of the inoculum removed from infected cells indicated that approximately 30% of the virus or 6 PFU/cell was adsorbed. At various times PI maintenance medium was removed and MEM plus 2% FBS plus 10 μ Ci of ³H-thymidine per ml (New England Nuclear Corp.) was added and cells were incubated at 35 C for 30 min.

Incorporation of radioactive uridine. Cell monolayers in roller bottles were infected with 20 PFU/cell, adsorbed 2 h at 37 C, inoculum was removed and

washed off; then cells were overlaid with MEM plus 2% FBS. After various intervals of incubation at 35 C, overlay medium was replaced with medium containing 10 μ Ci of 3 H-uridine per ml (New England Nuclear Corp., 0.0144 mg/5 ml) or 14 C-uridine per ml (53.1 mCi/ μ mol, Swartz Bioresearch). After incubation at 35 C for 20 min or 2 h, as indicated in Results, cells were chilled and washed in cold PBS.

Cell fractionation. Cells were scraped from the glass in cold PBS and centrifuged to pellet. Cells were then resuspended in 2 ml of RSB (0.01 M Tris, pH 7.4; 0.01 M KCl; 0.0014 M MgCl_2) for 2×10^7 cells and allowed to swell for 10 min at 0 C. Cells were then homogenized with 20 strokes in a Dounce homogenizer which resulted in cell breakage but did not disrupt the nuclei. The homogenate was centrifuged at $1,000 \times g$ for 5 min. The supernatant fluid contained the cytoplasmic fraction.

Determination of 3 H-thymidine incorporated into DNA. Cytoplasmic fractions were precipitated in 10% trichloroacetic acid, collected on Millipore filters and counted in PPO-POPOP scintillation fluid in a Packard Tri-Carb liquid scintillation spectrometer.

Coating of viral DNA. Overlay maintenance medium added after virus adsorption contained 10 μ Ci of 3 H-thymidine per ml. Samples were taken daily and cytoplasmic fractions isolated. Radioactivity of acid-insoluble material was determined in DNase treated and untreated samples. The ratio of DNase resistant material to total incorporated radioactivity was used as an indication of the percent of viral DNA coated.

Size distribution of RNA species in cytoplasm of infected and uninfected cells. 14 C-uridine-labeled cytoplasmic extracts were brought to 1% with respect to sodium dodecyl sulfate (SDS) then layered over a 26-ml 15 to 30% (wt/wt) linear sucrose gradient containing 0.5% SDS, 0.005 M Tris (pH 7.3), and 0.1 M NaCl. Uninfected Jinet cell RNA was used as a sedimentation marker in gradients containing labeled, infected cell, cytoplasmic fractions. Gradients were centrifuged and fractions were collected from the bottom of the tube in 0.8-ml amounts after passage through a Sigmamotor pump. Adsorbancy was determined with a Gilford spectrophotometer. Acid-insoluble radioactivity was precipitated from each fraction by addition of an equal volume of 20% trichloroacetic acid and collected on Millipore filters. Filters were washed with 10 ml of 10% trichloroacetic acid, dried, and counted in 10 ml of toluene scintillation fluid containing 0.5% PPO and 0.03% dimethyl-POPOP in a Packard Tri-Carb liquid scintillation spectrometer.

Extraction of cytoplasmic RNA. RNA was extracted from the cytoplasmic fraction as described by Oda and Joklik (16). The preparation was made 1% with respect to SDS and 0.2 M with NaCl. The mixture was then made 67% with respect to ethyl alcohol and stored overnight at -18 C to precipitate RNA-containing complexes. Precipitates were centrifuged down at 2,500 rpm for 20 min and resuspended in sodium acetate buffer. SDS was added to a final concentration of 1% and sodium perchlorate to 0.5 M. RNA was twice extracted in an equal volume of chloroform-isoamyl alcohol (24:1). The preparation

was then made 0.2 M with sodium chloride and 67% with respect to ethyl alcohol. RNA was stored at -18 C. The concentration was estimated spectrophotometrically, 1 OD unit at 260 nm corresponding to 45 μ g of RNA/ml (16).

Preparation of polysomal RNA from virus-infected cells. The cytoplasm was extracted from infected 3 H-uridine-labeled cells by the method of Borum et al. (3) by using a nonionic detergent, Nonidet P-40 (NP-40) (Shell Chemical Co.). Cells were suspended in isotonic buffer (0.14 M sodium chloride, 0.01 M Tris, pH 7.4, 0.0015 M magnesium chloride) and NP-40 was added to a final concentration of 0.5%. The mixture was incubated in ice for 15 min then centrifuged at 1,000 rpm for 5 min. The cytoplasm was contained in the supernatant fluid. Polyribosomes were isolated as described by Huang et al. (10). Cytoplasmic fractions were layered on 5 to 20% linear sucrose gradients, each formed over a 70% sucrose cushion and centrifuged at 17,500 rpm at 4 C for 5.5 h. The polysomes were collected and made 1% with SDS and allowed to stand at room temperature for 1 h. RNA was then extracted with chloroform-isoamyl alcohol as described above and precipitated with ethyl alcohol at -18 C.

Extraction of viral DNA. Yaba virus DNA was extracted by a combination of methods described for vaccinia virus (11, 16). Virus purified through two bandings in 20 to 40% sucrose density gradients was defatted by incubation in absolute alcohol at room temperature. Flocculated virus was centrifuged down at 10,000 rpm for 20 min and washed in 1:1 alcohol-ether. Virus was centrifuged again and washed in $1 \times \text{SSC}$ (0.15 M sodium chloride—0.015 M sodium citrate, pH 7). After centrifugation the pellet was resuspended in 0.3 ml of $1 \times \text{SSC}$ for 6 mg of virus. A 0.02-ml amount of 2-mercaptoethanol (14 M) was added, and the virus was incubated at room temperature for 1 h. A 0.2-ml amount of Pronase solution containing 2 mg/ml which had been preincubated at 37 C for 2 h was added to the virus preparation which was incubated overnight at 37 C. SDS was added to a final concentration of 0.5%, and the preparation was incubated at 37 C for 1 h. DNA was extracted with an equal volume of phenol saturated with 0.15 M sodium chloride—0.1 M ethylenediaminetetraacetic acid at pH 8.0. The extraction was performed by gentle rotation in an Erlenmeyer flask for 10 min. The preparation was then centrifuged at 200 rpm for 3 min. The aqueous phase containing the DNA was removed with gentle suction with a pipette which had had the tip sawed off to make a 3-mm bore. Phenol extraction was repeated. The DNA was then dialyzed against three changes of 1 liter of SSC. The first dialysis was 4 h in $0.1 \times \text{SSC}$, the second was overnight in $1 \times \text{SSC}$, and the third was 4 h in $1 \times \text{SSC}$.

Estimation of quantity of viral DNA. The quantity of DNA was estimated by the color reaction of Burton (4). Standard samples of calf thymus DNA and extracted viral DNA were mixed with diphenylamine reagent and incubated overnight. The OD₂₆₀ readings were taken in a Gilford spectrophotometer and a graph was plotted for the standards. The readings of viral DNA samples were interpolated on the

graph to determine the DNA concentration of each sample.

Denaturation of viral DNA. Approximately 100 μ g of DNA was diluted to 5 ml in $0.1 \times$ SSC. Two or three drops in 1 N sodium hydroxide were added to adjust the pH to 12.5. The preparation was hand-stirred for 10 min in ice. Two or three drops of 1 N hydrochloric acid were added to adjust the pH to 7.0.

Immobilization of viral DNA on filters. Nitrocellulose membrane filters (Schlacher and Scheull, Inc.) were presoaked in $6 \times$ SSC for 30 min then placed on a filtron suction apparatus. Denatured DNA was diluted in $6 \times$ SSC so that 10 ml contained 1 μ g of DNA. Samples of DNA (10 ml) in solution were placed on each filter with gentle suction. Blank filters received 10 ml of $6 \times$ SSC. Filters were dried overnight at room temperature then 4 h at 80 C in vacuo. Filters were stored in a dessicator.

Hybridization. DNA-RNA hybridization was carried out essentially as described by Gillespie and Spiegelman (8). RNA precipitates stored in 67% alcohol were centrifuged down at 13,000 rpm for 30 min at 4 C. RNA was dissolved in $2 \times$ SSC containing 0.1% SDS. RNA was quantitatively estimated by reading OD₂₆₀ in a Beckman spectrophotometer. One OD₂₆₀ was taken to equal 45 μ g of RNA (16). DNA-containing and blank filters were placed in scintillation vials and appropriate concentrations of RNA diluted in 1 ml of $2 \times$ SSC containing 0.1% SDS were added. Vials were incubated at 66 C for 22 h. Filters were rinsed through three beakers of 300 ml of $2 \times$ SSC, mounted on the filtron, and washed with 50 ml of $2 \times$ SSC with a slight vacuum. Filters were then incubated in 20 μ g of RNase per ml for 2 h. Filters were again washed in $2 \times$ SSC on the filtron and dried at room temperature overnight. Radioactivity was counted in Gentry's liquid scintillation fluid (7) in the Packard spectrometer.

RESULTS

Single-step growth curve. Cells infected with 20 PFU/cell were harvested at various intervals and titrated by the plaque method devised for Yaba virus (20). New progeny virus first appeared at 24 h PI and increased until 72 h, at which time the curve reached a plateau (Fig. 1).

Viral DNA synthesis. A plot of counts per minute of ³H-thymidine incorporated into acid-insoluble fraction in the cytoplasm of Yaba virus-infected cells versus time is shown in Fig. 2. The data indicate that DNA synthesis begins 12 to 14 h PI and reaches a peak at approximately 18 h. In uninfected cells no appreciable radioactivity was detected in the cytoplasm, indicating that no significant release of nuclear DNA occurred during isolation of the cytoplasmic fractions.

Coating of DNA. The coating of DNA as indicated by resistance to DNase during the first 7 days after infection is shown in Fig. 3.

Fig. 1. Single-step growth curve. Jinet cells were infected with 20 PFU/cell of purified Yaba virus in presence of 0.01 mM MgCl₂ and allowed to adsorb at 37 C for 2 h. Overlay MEM plus 2% FBS was added and incubation temperature was reduced to 35 C. At various intervals samples (approximately 1.7×10^7 cells) were harvested by freezing and thawing. Sonic treatment for three 1-min intervals was used to release intracellular virus. Titration was made by the plaque method for Yaba virus (20).

Maximum coating of the DNA occurred 4 days PI.

Sedimentation of RNA in the cytoplasm of infected and uninfected cells. Several studies (9, 13, 14) have shown that when ¹⁴C-uridine is used as a precursor to label RNA the only cellular RNA which reaches the cytoplasm in the first 30 min after label is 4S RNA. Infected and uninfected cells were labeled with ¹⁴C-uridine (10 μ Ci/ml) for 20 min at 37 C. The cytoplasmic fraction was separated from the cell nuclei and rendered 1% with respect to SDS. The size, distribution, amount, and incorporation of label was then analyzed by sucrose density gradient centrifugation. The OD₂₆₀ of each fraction was determined on the Gilford spectrophotometer, and the acid-insoluble radioactivity was precipitated with 10% trichloroacetic acid onto Millipore filters counted in PPO-POPOP liquid scintillation fluid. In experiments with uninfected cells all the radioactivity was distributed in the top of the gradient corresponding to the location of 4S RNA. Approximately 200 counts/min were obtained in uninfected cells. Neither of the two major OD₂₆₀ peaks on the gradient which represented the 28 and 16S RNA were significantly labeled during the 20-min exposure to ¹⁴C-uridine.

FIG. 2. Time course of DNA synthesis. Infection and incubation were carried out as described in Fig. 1. At various intervals overlay MEM was removed and MEM plus 2% FBS plus $10 \mu\text{Ci}$ of ^3H -thymidine per ml was added and incubation was continued at 35°C for 30 min. Cytoplasmic fractions from samples containing approximately 1.7×10^7 cells were obtained by Dounce homogenization. Radioactivity of acid-insoluble material was measured after precipitation in 10% trichloroacetic acid and collection was made on Millipore filters.

The results obtained with infected cells are shown in Fig. 4. At 6 h PI a species of rapidly labeled RNA ranging from 7 to 10S was being synthesized. At 12 h this peak as well as the 4S RNA increased in magnitude. By 24 h PI the rapidly labeled RNA was contained in fractions equivalent to 14 to 15S as well as 7 to 10S.

Hybridization of Yaba virus DNA with polysomal RNA as a function of time of incubation. A blank filter and six filters containing $1 \mu\text{g}$ each of Yaba virus DNA were placed in a vial containing $800 \mu\text{g}$ of polysomal Yaba virus ^3H -RNA labeled 11 to 13 h PI. The vial was then incubated at 66°C . At various times membranes were removed, washed, and incubated in RNase to remove RNA molecules which were not hybridized with the DNA contained on the filter. Filters were again washed, then counted in liquid scintillation fluid. A plot of counts versus time indicated that hybridiza-

tion occurred progressively with time and was essentially complete in 22 h, so this was the time allowed in succeeding experiments.

Amount of hybrid formed versus amount of viral RNA added. In order to determine whether or not the amount of hybrid formed in this system was a linear function of the amount of viral RNA added, polysomal RNA was extracted from infected cells labeled for 30 min at 12 h PI. This preparation was assumed to be as nearly free of cellular RNA as would be obtainable. Samples of 40, 80, 160, and $320 \mu\text{g}$ were hybridized with DNA filters punched out by a paper punch from a 25-mm filter containing $5 \mu\text{g}$ of viral DNA. A plot of counts obtained versus concentration of RNA was approximately linear.

Saturation of Yaba viral DNA as a function of concentration of RNA. In order to obtain a quantitative determination of virus-specific RNA by DNA-RNA complex formation, it is necessary that two conditions be met: (i) RNA must be present in lower than saturating

FIG. 3. Coating of DNA. Infection, adsorption, and incubation were conducted as described in Fig. 1, except that overlay medium added after adsorption contained $10 \mu\text{Ci}$ of ^3H -thymidine per ml. Cytoplasmic fractions were obtained by Dounce homogenization. Samples (1 ml) were incubated for 30 min at 37°C with 1 ml of $200 \mu\text{g}$ of DNase per ml. The radioactivity of these and 1-ml untreated samples was measured as described in Fig. 2. The ratio of counts in DNase treated to untreated samples was used to calculate the percentage coated.

FIG. 4. Sedimentation of rapidly labeled RNA. Approximately 2×10^7 cells were infected with 20 PFU/cell and pulse labeled with $10 \mu\text{Ci}$ of ^{14}C -uridine for 20 min at specific times after infection. Cytoplasmic fractions obtained by Dounce homogenization were made 1% with respect to SDS, layered on 15 to 30% (wt/wt) SDS-sucrose gradients, and centrifuged 18 h at 23,000 rpm at 20 C in an SW25 rotor. Fractions were collected from bottom of tube, precipitated with trichloroacetic acid, collected on Millipore filters, and radioactivity was measured. A, 6 h; B, 12 h; C, 18 h; D, 24 h.

levels and (ii) DNA must be present in excess (5). These parameters were determined for the Yaba viral DNA-RNA system. A standard quantity of poxvirus DNA previously shown (16) to be sufficient to saturate a 25-mm membrane filter of the type used in these experiments, namely $1 \mu\text{g}$, was placed on each filter. Filters were then incubated in vials containing various increasing concentrations of polysomal ^3H -RNA extracted from the cytoplasm of infected cells labeled 11 to 13 h PI. After 22 h of incubation at 66 C, filters were washed, digested with RNase, washed again, and counted. Counts per minute were taken to reflect the quantity of RNA forming an RNase-resistant complex with the DNA on the filter. The curve obtained indicated that $300 \mu\text{g}$ of RNA saturated the $1 \mu\text{g}$ of DNA on the filter, as evidenced by the plateau. Therefore, RNA was quantitatively estimated in the following experiment and sufficient DNA was provided so that no

more than $200 \mu\text{g}$ of RNA was present for $1 \mu\text{g}$ DNA.

Time of appearance of virus-specific RNA. Cells infected with a multiplicity of 20 PFU/cell were pulsed 2 h with $10 \mu\text{Ci}$ of ^3H -uridine per ml at various times PI. Cytoplasmic RNA prepared from approximately equal cell samples was quantitatively estimated. Duplicate membrane filters each containing $1 \mu\text{g}$ of YV DNA and a blank filter were incubated at 66 C in 1 ml of ^{14}C -RNA containing no more than $300 \mu\text{g}$. Results indicating the counts per minute hybridized at each interval are shown in Fig. 4. Virus-specific RNA present at each time interval was indicated by the amount of complex formation which was reflected by the counts per minute. A comparison of these estimations indicates that the peak of virus-coded RNA synthesis occurred at 11 to 13 h PI, and was followed by a second peak slightly less in quantity at 21 h.

In view of the existing possibility that changes in the synthesis of cellular RNA during the course of infection were so drastic as to affect the ratios in Fig. 5, an experiment was conducted in an attempt to confirm that the data was representative of the time course of viral RNA synthesis in these cells. Infected cells were labeled with ^{14}C -uridine for 20 min, and cytoplasmic fractions were isolated and acid-insoluble radioactivity was precipitated and counted as described for determination of incorporation of ^3H -thymidine. A plot of counts per

FIG. 5. Time of appearance of virus-specific RNA. Two filters each containing $1 \mu\text{g}$ of viral DNA and a blank filter were incubated at 66 C for 22 h in 1 ml of ^3H -polysomal RNA ($300 \mu\text{g}$ or less) obtained from 2×10^7 cells labeled for 2 h in $10 \mu\text{Ci}$ of ^3H -uridine per ml. Hybridized counts were determined as described in Fig. 2.

minute versus time resembled the pattern of viral RNA synthesis indicated in Fig. 5.

DISCUSSION

The growth curve obtained for Yaba virus by the plaque method is comparable to that reported by Yohn et al. (24) using focus formation as a means of titration. The first cycle of replication is about three times slower than that of vaccinia virus (21). Results reported herein agree with those obtained by Yohn et al. (24) with autoradiography and indicate that the time course of synthesis of Yaba virus DNA follows a pattern similar to that reported for other poxviruses. However, the entire pattern is considerably slower; vaccinia virus DNA is synthesized from 2 to 5 h PI (17).

Transcription of vaccinia virus DNA has been studied extensively. The virus replication cycle is divided into early and late phases. The early portion precedes DNA replication and lasts for about 1.5 h; the late portion follows until maturation of progeny is complete (11). Data obtained with sedimentation studies with Yaba virus (Fig. 4) suggest that two or more sizes of RNA are transcribed. The smaller species is most prevalent at 12 h PI. Assuming that the early and late replication cycle occurs with Yaba virus the data may be interpreted that significant amounts of progeny DNA are not yet available at 12 h for transcription of the larger species. By 24 h PI a species of 16S or larger RNA is being transcribed. Apparently, the amount of mRNA synthesized early exceeds the amount synthesized later in Jinet cells. This situation is comparable to that with vaccinia virus in L cells (16). The results indicate that the functional life of the smaller mRNA molecules synthesized at 12 h PI is not as great as that of the larger molecules synthesized later in the infection cycle.

Oda and Joklik (16) have concluded that the patterns and rates of mRNA transcription by vaccinia virus are influenced by multiplicity of infection. In experiments with poxviruses designed to study the time course of synthesis of nucleic acids the multiplicity of infection is generally quite high—approximately 100 infectious units per cell. In the studies reported herein the multiplicity of infectious particles per cell was lower (about six) although the number of virus particles per cell was high. This difference was necessitated by the peculiarity of Yaba virus which replicates to form a high ratio of noninfectious to infectious progeny (25). The condition of high multiplicity of infection is not readily attainable because when attempts are made to increase the inoculum beyond 20 PFU/

cell more than 10,000 particles/cell are required and the efficiency of adsorption is sharply reduced (unpublished data). The use of inoculum containing 10,000 particles per cell was the most efficient for adsorption (approximately 30%) under the conditions described. Attempts are being made to produce Yaba virus under various conditions which will yield increased ratios of infectious to noninfectious particles in order to study the transcription of mRNA after increased multiplicity of infection.

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