RNA Synthesis in Cells Infected with an Icosahedral Cytoplasmic Deoxyvirus (Frog Virus 3)

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Received for publication 24 January 1974

In cells infected with frog virus 3 there was extensive but not complete inhibition of host RNA synthesis. Host RNA processing was not impaired. The electrophoretic pattern of frog virus 3 specific RNA transcripts in infected FHM cells has been determined. When analyzed by gel electrophoresis, the transcripts separated in a size range of 6 to 16S with the bulk migrating around 16S. These RNA's contain polyadenylic acid stretches of approximately 150 to 200 nucleotides. In the absence of viral DNA replication, the rate of transcription is reduced, but the molecular size species correspond to those produced in normal infection. No evidence of distinct "early" RNA transcripts could be found.

Frog virus 3 (FV₃) is an icosahedral deoxyvirus (14) that replicates in the cell cytoplasm (6, 19). Its site of replication and genome composition (double-stranded DNA, 130×10^6 daltons; 25) invites comparison with the biologically unrelated poxviruses. The programming of DNA synthesis by FV₃ and poxvirus differs mainly in that FV₃ DNA synthesis continues throughout onset of the virus maturation phase, whereas poxvirus DNA synthesis reaches a peak and declines prior to the appearance of progeny virus (17, 18).

Poxvirus transcription has been studied extensively. The main features are biphasic transcription coupled to DNA synthesis (17, 18) and the presence of a virion-associated transcriptase that will synthesize early mRNA in vivo and in vitro (12, 13). We were interested to know if the FV₃ transcriptional system was similar to that of poxvirus or if it exhibited entirely novel modes of control. This communication describes some general aspects of FV₃ transcription.

MATERIALS AND METHODS

Virus. Crude FV₃ stocks were grown in FHM monolayers (8). Confluent monolayers were infected (MOI = 0.1 or less) and incubated at 28°C for 72 h. Cells were harvested, washed in phosphate-buffered saline (PBS), and disrupted by sonic vibration, and cellular debris was removed by centrifugation (10 min, $2,500 \times g$). The crude supernatant was layered over 30% (wt/vol) sucrose in $1 \times$ standard reticulocyte swelling buffer (RSB) and centrifuged (30 min, 25,000 rpm in an SW27 rotor). The pellet was diluted approximately 100-fold into PBS and was stored at

-70°C in ampules. Stocks used in these experiments contained 2×10^9 to 10^{10} PFU per ml (16).

Electrophoresis. Unless otherwise specified, all electropherograms refer to 1.7% acrylamide, 0.5% agarose gels prepared according to Peacock and Dingman (21). Running buffer was 0.1 M Tris-borate, pH 8.3, 2.5×10^{-3} M EDTA. Gels were 13 cm in length and were run approximately 3.5 h at 5 V/cm. A tracking dye, bromophenol blue, was used and gels were aligned relative to the dye position. RNA samples were applied in 12% glycerol $0.4 \times$ running buffer with or without dimethyl sulfoxide (DMSO, Fisher Scientific Co.) as stated. Gels were cut into approximately 2-mm slices and were incubated 90 min at 60°C with 0.5 ml of a mixture of 9:1:10 protocol (New England Nuclear Corp.), water, and toluene. The digested material was then chilled for 1 h at -20°C prior to counting in toluene-based scintillation fluid (Spectrafluor, Amersham). RNA could be extracted from gel slices with greater than 90% efficiency by shaking the fractions in 1 ml of TSM buffer (0.01 M Tris, pH 8.0, 0.1 M NaCl, and 0.001 M MgCl₂) at 37°C for 4 to 5 h.

Hybridization. Hybridization was performed at 66°C in $4 \times$ saline-citrate (SSC, 0.6 M NaCl, 0.06 M sodium citrate) 0.1% sodium dodecyl sulfate (SDS) for 24 h by using DNA trapped on filters (27 mm type B-6, Bac-T-Flex, Schleicher & Schuell, Inc.) as described by Green et al. (9); filters contained 20 µg of the indicated DNA.

DNA. FV₃ DNA was extracted from virus purified according to Tan and McAuslan (27). Purified virus in $1 \times$ RSB were exposed to DNase I (100 µg/ml) at 28°C for 30 min. The virus was then rebanded in a 20 to 55% sucrose gradient (27), and the viral band was concentrated by centrifugation through 30% sucrose (see above). The viral pellet was resuspended in $1 \times$ RSB containing 0.02 M EDTA and 0.1% SDS. Self-digested Pronase was added (1 mg/ml) and digestion

was allowed to proceed for 30 min at 37 C prior to a second addition of an equal amount of Pronase. After another 60 min, the DNA was extracted with an equal volume of cold phenol chloroform (1:1). The aqueous phase was re-extracted with the phenol-chloroform mixture, and the final supernatant was extracted twice with ethyl ether. DNA was precipitated in 70% ethanol and was exhaustively dialyzed against 0.1 M SSC prior to use in hybridizations.

FHM DNA. Nuclei were prepared from FHM (minnow) monolayer cells (8) by the NP₄₀ method detailed below. DNA was extracted from the isolated nuclei by the same method used to isolate DNA from FV₃ except that the nuclei were not exposed to DNase digestion.

Hybridization in formamide. Hybridization in 50% formamide was performed exactly as described by Bachenheimer and Roizman (2).

Infection of cells. Unless stated to the contrary, all infections were carried out at an input multiplicity of 20 PFU/cell on monolayers. Monolayers were grown in plastic flasks at 28 C in Eagle minimal essential medium (F-11 Grand Island Biological Co., N.Y.) with added glutamine and 10% inactivated fetal calf serum. The cells were drained and washed in fresh medium containing 0.02 M MgCl₂ and 2% inactivated fetal calf serum. Virus was applied in a small volume of this adsorption medium and allowed to adsorb for 1 h. After adsorption, the inoculum was removed and fresh medium was added.

Labeling of infected cells. At the indicated times after adsorption, samples were incubated with tritiated uridine or adenosine (10 μ Ci/ml, New England Nuclear Corp.) for the indicated labeling periods. Incorporation was stopped by the addition of excess cold precursor, and the cells were placed on ice. Monolayers were removed with a rubber policeman. Cells were collected and centrifuged at 600 $\times$ g for 4 min, and the pellet was washed in cold buffer A (0.01 M Tris, pH 8.5, 0.003 M CaCl₂, 0.01 M Mg acetate, 0.15 M KCl). At this point the cells were either fractionated into cytoplasm and nuclei or whole cell samples were disrupted by addition of SDS to 1% concentration. Whole cell lysates were then precipitated in 5% trichloroacetic acid and filtered. Extraction of RNA from whole cell samples by the hot phenol technique is described below. Cells to be fractionated were collected, suspended in cold NP₄₀ buffer (buffer A + 0.5% NP₄₀), and held at 0 C for 5 min. Under these conditions, cells lysed spontaneously with less than 1% of thymidine-labeled DNA appearing in the cytoplasmic fraction. The nuclei were removed by centrifugation (600 $\times$ g, 3 min), and the cytoplasmic fraction was brought to 1% SDS (see below for extraction of RNA). The nuclei were also lysed in 1% SDS and both fractions were precipitated in 5% trichloroacetic acid and filtered on GF/C glass filters. The air-dried samples were digested and counted as described for acrylamide gel slices.

Preparation of RNA from whole cells. Infected cells were labeled, collected, and washed as described above. Immediately after washing in cold buffer A, the cell pellet was resuspended in pH 5.0 acetate buffer (0.05 M). The cells were lysed in SDS (1%) and

were extracted in hot phenol as described by Scherrer (24). After ethanol precipitation, the pellet of purified RNA was digested with RNase-free bovine pancreatic DNase I (Worthington, 25 μ g/ml) at 37 C for 30 min. The reaction mixture contained 0.01 M Tris, pH 8.0, 0.04 M NaCl, and 0.01 M MgCl₂. RNA was re-extracted at 24 C with an equal volume of phenol and chloroform and was re-precipitated in ethanol. Between 65 and 80% of the incorporated uridine label was recovered by this method.

Preparation of RNA from cytoplasmic extracts. The supernatant from cytoplasmic fractions containing 1% SDS was extracted with an equal volume of phenol and of chloroform. Phases were separated after extraction by centrifugation at 5 C for 15 min at 4,400 $\times$ g. Interphase material was re-extracted twice with fresh buffer A and the aqueous extracts were combined. RNA was precipitated by addition of 3 to 4 volumes of ethanol and allowed to stand at -4 C for at least 24 h. Unless otherwise stated, cytoplasmic viral DNA was removed by LiCl₂ precipitation. The RNA in ethanol was collected by centrifugation at 4,500 $\times$ g for 30 min. The precipitate was resuspended in 0.01 M Tris, pH 8.0, and brought to 2 M LiCl₂. The RNA was allowed to precipitate for 12 h at 4 C. The precipitate was spun down at 4,500 $\times$ g for 30 min and was resuspended in 0.01 M Tris, pH 8.0, and was again precipitated in ethanol and stored at -20 C prior to use.

Binding to polydeoxythymidylate-cellulose. Poly(dT) cellulose was kindly supplied by S. Kerwar. Purified RNA was applied to a column (1 by 4 cm) of poly(dT)cellulose in 0.01 M Tris, pH 7.5, containing 0.5 M KCl. The column was washed with 5-column volumes of this application buffer. The majority of the input RNA eluted in the application wash and contains ribosomal and transfer RNA as well as mRNA and mRNA fragments which lack polyadenylic acid sequences (1). The column was then washed (5-column volumes) with the same buffer containing 0.01 M KCl. Less than 3% of the input RNA was eluted in this wash. The final wash was with buffer without salt, and the RNA was eluted which bound to the poly(dT)cellulose due to polyadenylic acid sequences. In the first wash, 1 to 2% of the total adenosine-labeled RNA was resistant to RNase digestion at high salt. The second wash contained 0.3 to 0.8% and the final wash contained 11 to 12% RNase-resistant RNA. RNase digestion was carried out at 37 C for 30 min in 0.32 M NaCl, 0.01 M EDTA with 14 units of RNase T1 per ml (Miles Laboratories) and 3.2 μ g of RNase A per ml (Seravac Laboratory).

RESULTS

Effect of FV₃ infection on host RNA synthesis. A number of papers have drawn attention to the observation that FV₃ infection of cultured cells caused a rapid inhibition of host RNA synthesis (10). As FV₃ appears to replicate in discrete cytoplasmic factories (15, 19), incorporation of tritiated uridine into the nuclear fraction of infected cells during a brief exposure period gives an approximate measure

of host RNA synthesis, and incorporation into the cytoplasmic fraction is indicative of the rate of viral RNA synthesis. The rate of nuclear RNA synthesis in infected FHM monolayers declines approximately 80% within 4 h after adsorption of FV₃, whereas incorporation into the cytoplasmic fraction increases (Fig. 1). However, although inhibition of synthesis is significant, nuclear RNA production is not completely eliminated in the infected cells. Furthermore, the rate of the residual nuclear RNA synthesis is 10 to 20% of the rate of the total RNA synthesis. These preliminary experiments indicate that, although host RNA synthesis is inhibited to a great extent, the residual synthesis of host species occurs at a significant rate relative to viral RNA synthesis. Furthermore the processing of host RNA to 28 and 18S species was not affected by FV₃ infection (data not shown).

Viral RNA in FV₃-infected cells. Since labeled host RNA will contribute significantly

to the overall species of RNA isolatable from infected cell, it was important to measure contamination by host RNA and to positively identify viral species by hybridization. Contamination by host species was successfully reduced by labeling for only a brief period and removing nuclei intact from the disrupted infected cells. RNA from the cytoplasmic extracts of briefly labeled infected cells form a series of peaks with the predominant species migrating at a position expected for a species with a sedimentation coefficient of 16S (Fig. 2). In contrast, much less RNA of the same size species was obtained from uninfected cells. Very similar patterns were obtained from infected FHM and BHK cells. Evidence for the viral origin of RNA in the 8 to 16S peaks was provided by hybridization of the RNA extracted from the gels (Fig. 3). Such RNA contained relatively little RNA hybridizable to host DNA (Fig. 4). This combination of a short-labeling period to reduce the time available for host RNA to be transported from the nucleus and separation of intact nuclei from the

FIG. 1. Effect of FV₃ infection on uridine incorporation into nuclear and cytoplasmic fractions. Infected FHM monolayers were labeled at the indicated times after infection for 15 min with tritiated uridine. Mock-infected controls which were labeled and sampled in parallel with the infected cells exhibited a constant ratio of cytoplasmic to nuclear incorporation although this ratio (C/N) varied from 0.4 to 0.8 in different experiments. Symbols: ○, cytoplasm; ●, nucleus.

FIG. 2. Electrophoresis of RNA from the cytoplasmic fraction of uninfected and FV₃-infected FHM cells. FHM monolayers were infected with FV₃. At 5 h postinfection, cells were labeled with tritiated uridine (50 μ Ci/ml) for 15 min. Monolayers were rinsed with cold PBS containing 0.01 M uridine, and cytoplasmic fractions were prepared. The cytoplasmic RNA was extracted and analyzed by acrylamide-agarose electrophoresis. An equal number of mock-infected cells were processed in the same way. Symbols: ○, RNA from infected cells; ●, RNA from mock-infected cells. The arrows indicate the position of ¹⁴C-labeled HeLa cell 28, 18, and 4S markers run simultaneously.

FIG. 3. Hybridization to FV_3 DNA of RNA extracted from acrylamide-agarose gels. Cytoplasmic RNA was prepared from FHM monolayers infected with FV_3 . The cells were labeled with tritiated adenosine for 30 min between 5.5 and 6 h postinfection. Purified RNA was resuspended from an ethanol pellet into 80% DMSO in $0.4\times$ running buffer prior to electrophoresis. The RNA from gel slices was extracted. RNase free DNase ($2\text{ }\mu\text{g/ml}$) was added to each fraction and allowed to incubate 30 min at 22°C to remove any trace amounts of DNA. Samples of 0.5 ml were removed from each fraction and precipitated in trichloroacetic acid (5%) and filtered ($\circ$). Fractions were pooled as indicated, re-extracted in phenol-chloroform (24°C), and precipitated in ethanol. The precipitated RNA was hybridized against FV_3 DNA. Background binding (120 to 231 counts/min) to blank filters is subtracted and data is presented as the percentage of input counts per minute hybridized (---) for the pooled fractions. No significant hybridization was found in fractions 1–10 and 11–19 (less than 0.5%). Arrows indicate position of [^{14}C]HeLa RNA marker species run simultaneously.

cytoplasm permits the isolation of labeled viral RNA with reduced levels of labeled host RNA.

“Early” viral RNA. In a number of deoxyvirus systems distinct “early” RNA species whose synthesis is independent of viral DNA synthesis are transcribed prior to initiation of DNA synthesis (see 17). To clearly separate any such species from “late” RNA, DNA synthesis was inhibited in order to permit transcription of only early species. In this system, DNA synthesis, as measured by a rise in thymidine incorporation into the cytoplasmic fraction starts at 3 h and continues for a further 12 h. When hydroxyurea or cytosine arabinoside was added to cultures prior to or at the time of infection, FV_3 DNA synthesis was inhibited (19); synthesis of viral-specific RNA was greatly reduced compared to normally infected cells, but virus-

specific RNA was synthesized over a long period of time (Fig. 5). When DNA synthesis was prevented indirectly by inhibition of protein synthesis (15), incorporation of uridine into RNA was markedly depressed (Fig. 6). This process was irreversible.

To compare the RNA species made, RNA from normally infected cells was compared with RNA from cells pretreated with hydroxyurea or cytosine arabinoside or cycloheximide (Fig. 7).

It can be seen that if viral DNA synthesis is prevented by hydroxyurea or cytosine arabinoside, the pattern of RNA synthesized in the cytoplasm is similar to that of normal viral RNA. There is no evidence of a distinct early RNA species, at least not in the size range 4 to 28S. The case of RNA made by cycloheximide-treated infected cells is not as clear due to the consistently low levels of incorporation. However, there appears to be a larger proportion of the RNA in the 4 to 10S region in the samples of cycloheximide-treated cells. Similar results

FIG. 4. Host RNA and viral RNA in briefly labeled cytoplasmic RNA of infected FHM cells. See legend to Fig. 3 for preparation of RNA. Increasing amounts of purified labeled RNA (994 counts per min per μg) were hybridized against duplicate filters of FHM, DNA, FV_3 DNA, and HeLa DNA (as a control) with $20\text{ }\mu\text{g}$ of DNA per filter. Backgrounds of 65 to 131 counts/min were subtracted. Symbols: $\bullet$, FV_3 DNA, $\circ$, FHM DNA.

FIG. 5. Effect of inhibition of DNA synthesis on viral RNA synthesis. FHM monolayers were infected with FV₃ at an MOI of 10. Immediately after the 1-h adsorption period, half of the monolayers were exposed to medium containing 1 μ Ci of tritiated uridine per ml and 10^{-4} M cold uridine. The other half received the same medium with 3.3×10^{-4} M hydroxyurea. At the times indicated, 1.5×10^6 cells were harvested from each group. The cells were not fractionated. The total cellular RNA was extracted by the hot phenol SDS method. The purified RNA was digested with DNase prior to hybridization against filters containing either FV₃ DNA (20 μ g) or no DNA. The hybridization was run in duplicate and at two different concentrations of RNA for each time sample. Each point is the average of duplicates at one RNA combination and is presented as the percentage of input RNA hybridized. The specific activity of the RNA samples was from 98.5 to 421.7 counts per min per μ g and the background was 46 counts/min average. The "zero" time point sample is RNA from uninfected cells, 0.11% of which bound to FV₃ DNA filters. Symbols: ○, infected cells without hydroxyurea; ●, infected cells plus 10^{-4} M hydroxyurea.

were obtained at high (100 μ g/ml) or low (10 μ g/ml) concentrations of cycloheximide.

As there is no biological assay to demonstrate the intactness of these RNA species, it is difficult to prove that they are not produced by degradation. The following experiment was designed to show that the cytoplasmic RNA (vi-

ral) species observed are stable within the infected cell and do not undergo extensive degradation in the presence of cytosine arabinoside.

Infected cells were briefly incubated with [³H]uridine late in infection. One sample was taken immediately after labeling, and the remainder were washed with medium containing excess cold uridine. Half of these remaining cells were allowed to continue the course of

FIG. 6. Effect of cycloheximide on RNA synthesis in infected FHM monolayers. Four sets of FHM monolayers were exposed to cycloheximide for 2 h and one group was not. All cells were infected with FV₃ at an MOI of 5 except for one control set of monolayers which was exposed to cycloheximide but not infected. [³H]uridine (1 μ Ci/ml) was added to all cells at 1 h postinfection. At the times indicated, duplicate monolayers were sampled, the cells were harvested, and acid-precipitable counts were determined from whole cell extracts. Symbols: ●—●, infected cells, no cycloheximide; □, infected cells, 10 μ g of cycloheximide per ml; ○, infected cells, 100 μ g of cycloheximide per ml; △, uninfected cells, 100 μ g of cycloheximide per ml; ●---●, infected cells, 100 μ g of cycloheximide per ml washed out at 2 h postinfection.

FIG. 7. FV_3 RNA from cells normally infected and exposed to hydroxyurea or cycloheximide. Hydroxyurea (10^{-4} M) was added to one set of FHM monolayers and cycloheximide (100 μ g/ml) to another 1 h before infection with FV_3 (MOI = 30). All three groups were infected, and at 8.5 h postinfection all were labeled with tritiated uridine (10 μ Ci/ml) for 15 min. Inhibitors were present continuously from the time of their addition. RNA was purified from cytoplasmic extracts and electrophoresed. Symbols: $\bullet$, normal RNA, specific activity 1,343.8 counts per min per μ g, 9.2% of input hybridized to FV_3 DNA filters; $\circ$, cells exposed to hydroxyurea, specific activity 618.1 counts per min per μ g, 4.6% of input RNA hybridized to FV_3 filters; Δ , cells exposed to cycloheximide, specific activity 169.0 counts per min per μ g RNA, 3.8% of input RNA hybridized.

infection in the presence of cold uridine while cytosine arabinoside was added to the other half. Addition of cytosine arabinoside to the infected cells rapidly inhibited incorporation of uridine from the internal pool, whereas the normally treated cells continued incorporation at a declining rate for at least 6 h (Table 1).

The pattern of viral RNA observed immediately after labeling (Fig. 8) was still present 6 h after the cold uridine chase. The only major difference is the appearance of 28S RNA in the cytoplasm as would be expected from the fact that the synthesis and processing of host RNA continues after infection. Analogous "pulse-chase" experiments using actinomycin D (10^{-4} M) to block RNA synthesis in place of cytosine arabinoside give similar results (data not shown).

Polyriboadenylic acid in viral RNA. It has

TABLE 1. Effects of cytosine arabinoside on incorporation into cytoplasmic RNA after briefly labeling infected cells^a

Time after addition of cold uridine (h)	- ARA-C ^b	+ ARA-C
0	40,101	38,509
1	190,172	202,885
4	209,689	301,896
6	194,089	343,550

^a See legend to Fig. 8. Cytoplasmic extracts (see Materials and Methods) were made from two monolayers for each time point. Net incorporation into the cytosine arabinoside-treated cells stopped within 1 h of addition of drug plus excess cold uridine. In the absence of cytosine arabinoside cells continued to incorporate label from the internal pool of uridine at a declining rate.

^b [³H]uridine incorporated into acid-precipitable counts per minute.

FIG. 8. Stability of viral RNA in the presence and absence of cytosine arabinoside. Infected FHM monolayers were incubated (15 min) with [3H]uridine, 6 h after infection. RNA was purified from cytoplasmic extracts from one third of the cells immediately after labeling. The remaining cells were washed with medium containing cold uridine (10^{-3} M), divided into two groups (with and without 10^{-4} M cytosine arabinoside), and were allowed to incubate for 6 h in medium containing cold uridine (10^{-3} M). During the 6-h incubation period after labeling, there was no decrease in incorporated label for either group as measured by acid-precipitable counts in whole cells from duplicate monolayers sampled at 1, 4, and 6 h after labeling (Table 1). Cytoplasmic RNA purified from the two groups of cells 6 h after labeling was compared with RNA isolated immediately after labeling by gel electrophoresis. HeLa cell RNA (^{14}C) was used as a size marker. The amount of label applied to gels for the three samples was: ●, RNA taken immediately after labeling, 29,000 counts/min applied; ○, RNA from cells incubated 6 h after labeling, no cytosine arabinoside, 50,000 counts/min applied; Δ, RNA from cells incubated 6 h after labeling, plus 10^{-4} M ARA-C, 40,000 counts/min applied.

been suggested that polyadenylic acid sequences at the 3' end of mRNA's might be involved in transport of RNA from the cell nucleus to the cytoplasm (5). However, poxvirus mRNA contains polyadenylic acid sequences which are unlikely to be involved in such a transport process. It was of interest to know if SV_3 RNA also contains polyadenylic acid sequences, particularly in view of the possibility that they might be synthesized by a virion-encapsulated enzyme as appears to be the case for poxvirus (3, 7).

The majority of SV_3 -specific RNA contains polyadenylic acid sequences of sufficient length to bind to poly(dT)cellulose. FHM monolayers were infected with SV_3 and labeled with either

tritiated uridine or adenosine for 15 min at 6 h postinfection. Purified RNA (10^6 counts/min) from the cytoplasmic fraction was passed over a poly(dT)cellulose column. The bound fraction was eluted (see Materials and Methods) and brought to $4\times$ SSC plus 0.1% SDS. Approximately 10% of this RNA was hybridizable to SV_3 DNA. Most (70%) of the hybridizable RNA was found in the fraction binding to dT-cellulose. Table 2 summarizes experiments which support the conclusion that at least some of the viral RNA contains polyadenylic acid. Furthermore, the RNase-resistant sequences (polyadenylic acid) occur in all size classes of viral RNA, although certain species contain more resistant material than others (Fig. 9). The RNase-resist-

TABLE 2. *Poly(A) in FV₃ RNA (%)*^a

RNA	Total counts/min	Acid-precipitable counts/min after RNase	Counts/min bound to dT-cellulose after RNase
[³ H]adenosine RNA	1.7×10^6	2.6×10^5 (15.1%)	2.2×10^5 (12.7%)
[³ H]uridine RNA	4.4×10^5	1.4×10^4 (3.2%)	9.2×10^2 (0.2%)
[³ H]adenosine RNA eluted from gel	2.0×10^4	2.4×10^3 (11.8%)	
[³ H]adenosine RNA eluted from hybrid	2.9×10^4		3.1×10^3 (10.8%)

^a Presence of poly(A) sequences in RNA from FV₃-infected cells. RNA was prepared at 6 h postinfection from the cytoplasm of FV₃-infected FHM monolayers after 15 min of labeling with either tritiated uridine or adenosine. Part was subjected to RNase digestion (37 C for 30 min with 1.6 μ g of RNase A per ml and 7 units of RNase T per ml, in 0.32 M NaCl, 0.02 M EDTA) and the RNase digest then passed over a poly(dT)cellulose column. Portions of bound material were hydrolyzed greater than 99% after 18 h in 0.6 M KOH at 37 C. Another portion of the labeled RNA was subjected to electrophoresis, and then eluted from gels and pooled (see legend to Fig. 9 for details of elution of RNA from gels). Such RNA labeled with tritiated adenosine was hybridized to FV₃ DNA (20 μ g/filter) in 50% formamide—5 $\times$ SSC at 43 C for 24 h (2). The hybridized viral RNA was eluted from washed filters in 0.01 M Tris, pH 7.5, 0.01 M EDTA by shaking for 1 h at 37 C. Of the input RNA (5×10^5 counts/min) 5.7% was recovered from hybrids. The eluent RNA also bound to poly(U) filters (36.3% of input) (23) prior to RNase digestion. The RNA was digested and was passed over poly(dT)cellulose by using the methods described above. Counts per minute refers to acid-precipitable counts.

FIG. 9. Distribution of RNase resistant RNA in viral RNA. RNA was purified from the cytoplasm of infected FHM monolayers after 15 min of labeling with tritiated adenosine. The sample was applied for electrophoresis in 80% DMSO—0.4 $\times$ running buffer. RNA from gel slices was eluted. The eluate was digested with DNase (2 μ g/ml) for 30 min at 25 C, and 0.5 ml was precipitated in acid (●). The remainder was brought to 0.32 M NaCl, 0.02 M EDTA, and digested with 3.2 μ g of RNase A per ml, 14 units of RNase T per ml at 37 C for 30 min followed by acid precipitation (○). Total recovery (%) of RNase-resistant counts per minute from the eluent material is given in Table 1 (third line).

ant material from FV₃ RNA has a size distribution which is about the same as that of a wide variety of other viral and cellular mRNA's (apparent size 150 to 200 nucleotides, cf. Fig. 10 and ref. 2).

DISCUSSION

One of the characteristics of FV₃ which has been singled out for attention is its ability to severely inhibit host cell function (4). Although

FIG. 10. Size of RNase-resistant viral RNA. RNA was purified from the cytoplasm of infected FHM monolayers after labeling 15 min with tritiated adenosine. The RNA was digested first with DNase, and then with RNase (see legend to Fig. 9), and was precipitated in ethanol with cold polyadenylic acid carrier. The RNA was denatured in 80% DMSO prior to application to acrylamide gels. Electrophoresis was carried out on 11-cm gels of 10% acrylamide at 150 V. Tritiated tRNA was run simultaneously as a size marker. The size of the RNase-resistant material was calculated from its migration relative to the 4S marker to be an average of 60,000 daltons, or about 175 nucleotides.

the virus does cause a rather rapid decrease in host cell RNA synthesis, residual RNA synthesis continues at a significant level. The infected cell continues to produce rRNA and mRNA and rRNA appears to be processed normally from precursor molecules (unpublished observations). FV₃ RNA is transcribed at a comparatively low rate, in fact about 1/10 of that of poxvirus in the same cell (data not cited). This makes it difficult to readily study the pattern of FV₃ transcription.

Because of the problems implicit in the cytoplasmic replication of nucleic acids, it is of interest to compare the modes of transcription of the two virus groups represented by poxvirus and FV₃. The transcriptional pattern of poxvirus can be understood in terms of the sequential events of uncoating (13, 17) and the presence of a virion-associated transcriptase (12). FV₃ appears to be uncoated in a single step independent of protein synthesis (25), and to date no virion RNA polymerase has been detected.

The "early" mRNA of poxvirus is transcribed

in a distinct wave of synthesis preceding DNA replication; this RNA is readily distinguished from "late" RNA species by size (13, 20). FV₃ RNA produced in cells infected in the presence of inhibition of viral DNA synthesis appears to be identical to RNA from normally infected cells. In agreement with this observation is a report that most, if not all, viral proteins are produced in the absence of viral DNA synthesis (R. M. Goorha and A. Granoff, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 248, 1973). Because of the high input multiplicity of infection and the efficiency of inhibition of viral DNA synthesis by cytosine arabinoside, it is unlikely that we were measuring RNA synthesized from "breakthrough" of some viral DNA synthesis. A very similar situation occurs in herpesvirus-infected cells where parental DNA is transcribed for the production of most viral structural proteins (22).

At least some FV₃ transcripts contain stretches of polyadenylic acid, and in this regard FV₃ resembles poxvirus and a great many other viruses. However, we were unable to detect any virion-associated enzyme which might give rise to these polyadenylic acid tracts when various assay methods described for poxvirus (3, 11) were applied to purified or crude virus.

FHM cells infected with FV₃ under conditions that permit extensive FV₃ replication (19) do not show a distinct burst of FV₃ RNA synthesis preceding DNA synthesis, nor can any such burst be magnified by infection in the presence of cycloheximide as is the case for poxviruses (17).

The very low rate of RNA synthesis in FHM cells pretreated with cycloheximide and infected with FV₃ precludes extensive studies and determination whether the 6S RNA species (Fig. 7) represents pre-early messenger. We can only point out that (i) it does appear to be the predominant species unlike the case for RNA produced in the presence of cytosine arabinoside, and that (ii) the available evidence on FV₃ uncoating which is not extensive (25) gives no indication of why RNA made in the presence of cycloheximide should differ from RNA made in the presence of cytosine arabinoside; currently there are no established early enzymatic markers of FV₃ that could be used to test the function of this RNA.

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