Nuclear Accumulation of Influenza Viral RNA Transcripts and the Effects of Cycloheximide, Actinomycin D, and α -Amanitin

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Received for publication 7 August 1978

The use of virus-specific ³²P-labeled complementary DNA and ¹²⁵I-labeled virion RNA as hybridization probes has allowed us to quantitate the number of molecules of complementary RNA (cRNA) and progeny virion RNA in MDCK cells infected with influenza virus. We compared the distribution of cRNA between the nucleus and the cytoplasm in cycloheximide-treated cells to that found in untreated cells, beginning 1 h after infection. A greater percentage of the total cRNA was detected in the nucleus of the drug-treated cells at all times investigated. For the first 2 h after infection about 50% of the cRNA synthesized in the cycloheximide-treated cells was found in the nucleus. These nuclear cRNA molecules were characterized and shown to be polyadenylated transcripts of each of the genome virion RNA segments. Viral cRNA synthesis was not completely inhibited by the addition of actinomycin D at the beginning of infection, with or without the concomitant addition of cycloheximide. A large fraction (about 90%) of these cRNA sequences were detected in the nucleus. Characterization of these nuclear cRNA molecules showed that they contained polyadenylic acid and represented transcripts of both those segments coding for proteins synthesized predominantly early after infection ("early" proteins) and those virion RNA segments coding for "late" proteins. Also, *in vitro* translation of these cRNA molecules showed that they were functional virus mRNA's. In contrast to actinomycin D, α -amanitin completely inhibited cRNA synthesis when added at the beginning of infection, and addition of this drug after 1.5 h had no effect on further cRNA synthesis.

The genome of influenza type A viruses consists of eight segments of single-stranded RNA molecules (15, 19, 25). The viral mRNA is complementary to the virion RNA (vRNA) (2-4, 24). The viral mRNA segments, whether synthesized *in vitro* or *in vivo*, contain polyadenylic acid [poly(A)] and are slightly smaller than the corresponding vRNA segments as a result of termination of transcription at a site 20 to 30 bases before the 5' end of the vRNA (5, 6, 22, 23, 27). Hay et al. (6) have shown that the infected cell also contains a second population of complementary RNA (cRNA) molecules which lack poly(A), are complete copies of each vRNA segment, and, they suggest, probably act as templates for progeny vRNA synthesis.

Although initiation of virus replication requires the host cell nucleus (8), it is not yet known whether cRNA, or vRNA, synthesis occurs in the nucleus and/or the cytoplasm. The presence of internal N⁶-methyl-adenosine residues in viral mRNA (11) is consistent with at

least some step(s) in the synthesis of these molecules occurring in the nucleus.

We have previously introduced the use of hybridization kinetic analysis to quantitate virus-specific RNA in infected cells (29). The infected cell nucleus was found to contain about 30% of the viral cRNA at early times (1.75 h). The proportion in the nucleus was increased when cycloheximide (CM) was added at zero time of infection to restrict cRNA synthesis to primary cRNA transcription. When both actinomycin D and CM were added at the time of infection, there was a substantial inhibition of cRNA synthesis, but of those cRNA molecules that were synthesized, the majority was detected in the nucleus. This finding is consistent with the hypothesis that primary transcription occurs, at least predominantly, in the nucleus.

The present study was initiated to extend our previous investigations of viral RNA transcription by determining the location of virus-specific cRNA at earlier times after infection and also to

characterize those molecules which accumulate in the nucleus in the presence of CM and/or actinomycin D. Also, in the hope of clarifying the contributory role played by the host cell in viral RNA transcription, we have compared the effects of α -amanitin and actinomycin D, two drugs that inhibit both cellular and viral RNA synthesis.

MATERIALS AND METHODS

Cells and virus. The procedures for the culture of the MDCK (canine kidney) cell line and for the growth and purification of WSN virus have been described previously (9, 10).

Preparation of infected cell cytoplasmic and nuclear RNA. MDCK cells were infected with WSN virus at a multiplicity of 30 to 60 PFU per cell (29). The virus was adsorbed at 4°C for 1 h. The inoculum was removed, and the cells were washed with viral growth medium to remove residual unadsorbed virus. The cells were then brought to 37°C by the addition of warm medium. Zero time corresponds to the time at which the cells were brought to 37°C. At the indicated times, the infected cells were collected and fractionated into nucleus and cytoplasm, and each fraction was extracted three times with phenol-chloroform at pH 9.0, as described previously (29). The ethanol-precipitated RNA was chromatographed through Sephadex G-50 in water to desalt the RNA. The RNA in the excluded volume was pooled and lyophilized. Where indicated, poly(A)-containing RNA was prepared by chromatography on oligodeoxythymidylic acid-cellulose (3).

Preparation of purified viral cRNA and vRNA. The procedures have been described for obtaining radio-pure viral cRNA from infected cells (3) and vRNA from purified virus (10). Individual influenza vRNA segments were separated by acrylamide slab gel electrophoresis. The vRNA had been labeled with [³H]adenosine during infection so that the purification of the segments could be monitored. Purified vRNA (250 μ g) was applied to a 2.8% polyacrylamide (20:1 acrylamide-bisacrylamide) slab gel (36 by 14 by 0.3 cm) containing 6 M urea in the buffer of Peacock and Dingman (20). After electrophoresis for 44 h at 140 V, the gel was stained with ethidium bromide (3 μ g/ml). Segments 1 to 3 were treated as a single pool. Thus, the gel slices containing this pool and those containing the individual segments 4, 5, 6, 7, and 8 were crushed in a phenol-saturated buffer containing 0.5 M LiCl, 0.5% sodium dodecyl sulfate, and 0.01 M Tris-hydrochloride (pH 7.4). The eluted RNA was extracted twice with phenol-chloroform (1:1) and three times with ether, and any remaining gel debris was removed by centrifugation (15,000 $\times$ g, 10 min). The RNA was ethanol precipitated and further purified by sedimentation for 26 h at 40,000 rpm in an SW40 rotor (Beckman) using a gradient of 15 to 30% sucrose in 0.1 M NaCl-0.001 M EDTA-0.01 M Tris-hydrochloride (pH 7.4).

Quantitative analysis of virus-specific RNA by determination of the kinetics of annealing of labeled probes. [¹²⁵I]vRNA (approximately 100 cpm/pg) and ³²P-labeled complementary DNA

(cDNA) (500 cpm/pg) probes for virus-specific cRNA and vRNA, respectively, were prepared as described before (29, 30). The cellular RNA sample to be assayed was suspended in water. A sample was used to determine its RNA concentration by the orcinol reaction. The RNA solution was heat denatured (95°C for 3 min) in a 0.15-ml conical tube, mixed with about 12,000 cpm each of [¹²⁵I]vRNA and [³²P]cDNA, and equilibrated at 68°C. Annealing and subsequent nuclease digestion were performed as described previously (29). Each set of annealing studies contained separate samples of purified vRNA and cRNA to determine the maximal extent of hybridization of the [³²P]cDNA and [¹²⁵I]vRNA probes, respectively.

The kinetics of annealing of the ³²P- and ¹²⁵I-labeled probes was entered into a Digital 11/70 computer programmed to determine the average number of molecules, per cell fraction, of each viral RNA species. It should be noted that although this publication is concerned almost exclusively with the number of cRNA molecules found in the infected cell, this number can only be accurately determined by hybridization when the number of vRNA molecules present is also ascertained (29).

In vitro translation of infected cell RNA. Oligodeoxythymidylic acid-cellulose-selected cytoplasmic or nuclear RNA was translated by an L-cell extract that had been pretreated with micrococcal nuclease to reduce endogenous protein synthesis (21). The assay conditions were as described elsewhere (1), using [³⁵S]methionine as a labeled precursor. The ³⁵S-labeled proteins were subjected to electrophoresis on 14% acrylamide-0.093% bisacrylamide gels with the Laemmli buffer system (12).

Materials. α -Amanitin was obtained from Boehringer-Mannheim, CM came from the Upjohn Co., and actinomycin D was from Merck, Sharp and Dohme. All radioactive precursors were obtained from New England Nuclear Corp.

RESULTS

Appearance of cRNA and vRNA in the nucleus and cytoplasm during infection; comparison of untreated and CM-treated cells. We previously reported that at 2.5 h after infection in CM-treated cells the number of cRNA molecules synthesized was reduced by 85% relative to untreated cells and that the percentage of the cRNA molecules detected in the nucleus increased relative to that observed in untreated, infected cells (18% versus 5%) (29). We have extended that experiment to study several time points from 1 to 3.5 h. Determination of the average number of molecules of viral cRNA and vRNA in the nucleus and cytoplasm of these cells was ascertained, as described in Materials and Methods, from the kinetics of annealing of radioactive probes to the extracted RNA (Fig. 1 and Table 1).

In cells not treated with CM, newly synthesized cRNA could be detected at 1 h after infection, with approximately 27% of these sequences

FIG. 1. Time course of appearance of cRNA and vRNA in the nucleus and cytoplasm of influenza virus-infected cells. The average number of molecules per cell fraction was determined by computer analysis of the kinetics of annealing of radioactive probes for cRNA and vRNA as described in the text. (a) cRNA appearance, (b) vRNA appearance, and (c) cRNA appearance in infected cells treated with 100 μ g of CM per ml from zero time. (O) Nucleus; (●) cytoplasm.

being located in the nucleus (Fig. 1a; Table 1). At later times, the number of nuclear cRNA molecules gradually increased, but the increase in cytoplasmic cRNA molecules was much greater, so that the nuclear cRNA represented only 5 to 10% of the total cRNA present in the cell. The increase in cytoplasmic cRNA molecules continued until 2.5 h. Between 2.5 and 3.5 h, we observed, as previously reported (29), a decrease in the amount of cytoplasmic cRNA. This decrease did not reflect a nonspecific loss of virus-specific RNA from the extracted RNA since vRNA sequences continued to accumulate during this time (see below). Examination of late time points with probes specific for segments 1 to 3, 5, 7, and 8 showed that the amounts of all of the above cRNA sequences decreased (data not shown). It is thus possible that at late times after infection the degradation of cRNA exceeded its synthesis.

After adsorption at 4°C (zero time), approximately 2,000 molecules of vRNA were detected in untreated, infected cells (Fig. 1b; Table 1). This vRNA derived from adsorbed inoculum virus appeared to be equally distributed between the nuclear and cytoplasmic fractions. The number of vRNA molecules decreased in the first hour after infection, and it was not until 1.5 to 2 h, or about 30 min after cRNA synthesis commenced, that newly synthesized vRNA could be detected. Between 2 and 3.5 h vRNA rapidly accumulated in the cells, especially in the cytoplasmic fraction. It should be noted that the fraction of the cRNA molecules that was located in the nucleus was greater before than after detectable vRNA replication occurred.

The residual cRNA synthesized in CM-treated cells was only 5 to 15% of controls, and no increase in vRNA molecules was detected. In fact, as a function of time after infection, the amount of vRNA molecules decreased. Newly synthesized cRNA molecules were detected by 1 h after infection, and their numbers increased for the next 2 h (Fig. 1c). The fraction of these cRNA primary transcripts detected in the nucleus was higher than that seen in untreated, infected cultures (Table 1). For the first 2 h, approximately 50% of the cRNA synthesized in CM-treated cells was detected in the nucleus.

Characterization of the primary transcripts located in the nucleus. We first undertook to determine whether the nuclear cRNA transcripts were of normal length. Since adenylation of cRNA is the terminal event in the synthesis of molecules destined to become mRNA, the presence of poly(A) on cRNA transcripts should be indicative of their normal length for viral mRNA. Thus, the RNA extracted from the nucleus and cytoplasm of CM-treated, infected cells at 2.5 h of infection was chromatographed on oligodeoxythymidylic acid-cellulose (Table 2). Of the primary transcripts found in the nucleus of CM-treated cells, 92% were adenylated, whereas slightly less of the cytoplasmic cRNA's (81%) contained poly(A). On the other hand, in control cultures where vRNA replication was in progress, only half of

TABLE 1. Content and distribution of cRNA and vRNA in influenza virus-infected MDCK cells^a

Treatment	Time of collection (h)	cRNA		vRNA	
		No. of viral genome equivalents	% Nuclear	No. of viral genome equivalents	% Nuclear
None	0	22		2,612	53
	1.0	298	27	821	44
	1.5	1,732	18	1,075	52
	2.0	8,682	7	2,806	31
	2.5	20,211	5	11,081	19
	3.0	9,692	13	15,903	22
CM	3.5	9,287	7	40,117	17
	1.0	180	38	1,256	65
	1.5	287	56	747	56
	2.0	267	44	643	38
	2.5	1,235	28	809	47
	3.0	2,131	25	761	44
	3.5	1,064	15	689	68

^a CM, when used, was present at 100 μ g/ml from zero time of infection. The fractionation of the infected cells and the determination of the number of genome equivalents are described in the text. These data are represented graphically in Fig. 1.

TABLE 2. Determination of the fraction of cRNA molecules that contain poly(A) sequences^a

Treatment	Nucleus			Cytoplasm		
	No. of molecules		% Poly(A) ⁺	No. of molecules		% Poly(A) ⁺
	Poly(A) ⁺	Poly(A) ⁻		Poly(A) ⁺	Poly(A) ⁻	
None	480	303	58	7,084	7,882	48
CM	1,293	111	92	1,006	237	81

^a CM, when used, was present at 100 $\mu\text{g}/\text{ml}$ from the time of virus adsorption ($t = -1$ h). At 2.5 h after infection the cells were fractionated. The extracted RNA was chromatographed on oligodeoxythymidylic acid-cellulose, and the number of genome equivalents was determined on the bound and unbound RNA, as described in the text.

the nuclear and cytoplasmic cRNA molecules were adenylated. Thus, we infer from these results that: (i) the primary transcripts present in the nucleus are of normal length for mRNA species, and (ii) the inhibition of protein synthesis by CM increased the percentage of cRNA molecules containing poly(A), consistent with the inhibition of the synthesis of the poly(A)-deficient cRNA molecules that serve as templates for vRNA synthesis.

We have also ascertained whether the cRNA in the nucleus of CM-treated cells is representative of all of the genome vRNA segments. To do this, vRNA segments were separated by electrophoresis (Fig. 2), as described in Materials and Methods, and used to make specific labeled probes. To measure the purity of each of the isolated vRNA segments, each [³²P]cDNA probe was hybridized to the vRNA segment used for its synthesis (homologous vRNA segment) (Fig. 3). The curves demonstrate sharp transitions, and the calculated $C_{t1/2}$ values were consistent with the known complexity of each RNA segment (7, 15). Several [³²P]cDNA's were hybridized to heterologous vRNA segments; the resulting kinetics of hybridization indicated at most a 5% contamination of one segment with another. We therefore concluded that the rate of annealing of the individual segment probes would provide an unambiguous measure of the number of each vRNA segment and its complement in an RNA fraction from infected cells.

These probes were used to quantitate the number of molecules of cRNA transcribed from each segment in the nuclei and cytoplasm of CM-treated cells (isolated at 1.8 h after infection). The primary transcripts in the nucleus, like those found in the cytoplasm, contained sequences complementary to each of the vRNA segments (Table 3).

Effect of the early addition of actinomycin D on viral RNA transcription and replication. We previously demonstrated that the addition of both actinomycin D and CM at zero time of infection reduced primary transcription to 15% of the untreated value, but, more impor-

FIG. 2. Preparative polyacrylamide slab gel electrophoresis of influenza virus genome segments. Virion RNA (250 μg) was applied to a 2.8% polyacrylamide slab gel containing 6 M urea and subjected to electrophoresis as described in the text. The gel was stained with ethidium bromide and photographed under UV illumination. The numbers refer to the eight genome segments.

FIG. 3. Annealing kinetics of individual [³²P]-cDNA probes to their homologous vRNA segments. [³²P]cDNA probes (10,000 cpm per reaction), prepared from each of the electrophoretically separated virion RNA segments shown in Fig. 2, were annealed in the presence of an excess of their homologous vRNA as described in the text. At the indicated values of $C_{t1/2}$, samples were removed and the formation of hybrids was assayed by resistance to nuclease S1. Each annealing curve, numbered with respect to the genome segment vRNA that was in excess, has been corrected for 100% annealing of the [³²P]cDNA probe to an excess of unfractionated vRNA. The actual extent of annealing of each probe varied between 75 and 95%.

tantly, the majority of the residual cRNA was found in the nucleus. We have now examined the effect of actinomycin D alone. Infected cell cultures were treated from the time of adsorp-

tion ($t = -1$ h) with actinomycin D and analyzed for the accumulation of virus-specific RNA sequences at 2.5 h after infection. For the purpose of comparison, analysis of CM-treated and untreated, infected cells was also performed. Although we were unable to detect any vRNA replication in the drug-treated cultures (data not shown), transcription of cRNA was present in all (Table 4). In the presence of actinomycin D (2 $\mu\text{g/ml}$) alone, transcription was reduced to 12% of that seen in the untreated cultures. Of the residual cRNA that was transcribed, 80 to 90% accumulated in the nucleus. This striking result was observed regardless of the dose of actinomycin D used for the concomitant presence of CM.

Characterization of the cRNA accumulating in the nucleus in the presence of actinomycin D. We sought to determine whether the cRNA accumulating in the nucleus of actinomycin D-treated, infected cells had the characteristics of authentic viral mRNA. Of the cRNA found in the nucleus in the presence of actinomycin D, with or without CM addition, 96% contained poly(A) (Table 5). These results suggest that the cRNA in the nucleus of actinomycin D-treated cells was of the normal size for viral mRNA.

TABLE 3. Average number of molecules of individual cRNA segments detected in CM-treated, infected cells^a

Cell fraction	No. of molecules in cRNA segment:					
	1,2,3	4	5	6	7	8
Nucleus	176	314	229	84	94	114
Cytoplasm	45	203	210	95	63	202

^a Infected cells, treated with 100 μg of CM per ml from zero time, were collected at 1.8 h and fractionated into nuclei and cytoplasm. The extracted RNAs were hybridized to probes specific for each genome segment, and the average number of cRNA molecules present was determined as described in the text.

TABLE 4. Average number of cRNA molecules accumulated in drug-treated cells at 2.5 h after infection^a

Drug treatment (concn in $\mu\text{g/ml}$)	No. of molecules in:		% Nuclear
	Cytoplasm	Nucleus	
CM (100)	837	594	42
CM (100) + AD (2)	81	312	79
None	8,102	861	10
AD (2)	125	959	89
AD (10)	62	538	89

^a CM and/or actinomycin D (AD) was added at the time of virus adsorption ($t = -1$ h). RNA was extracted from the cell fractions, and the average number of viral genome equivalents was determined as described in the text.

TABLE 5. Characterization of the viral cRNA molecules that accumulate in the nucleus of CM-plus-actinomycin D-treated cells^a

% Poly(A) ⁺	Avg no. of cRNA molecules in viral RNA segment:					
	1,2,3	4	5	6	7	8
96	200	— ^b	353	—	161	214

^a Infected cells were treated with CM (100 $\mu\text{g/ml}$) and actinomycin D (2 $\mu\text{g/ml}$) after adsorption ($t = 0$ h). RNA was extracted at 2.5 h from cells that had been fractionated into nucleus and cytoplasm. All determinations were as described in Tables 2 and 3.

^b —, Not assayed.

Using the probes specific for individual vRNA segments, we determined whether the nuclear cRNA contained transcripts for proteins synthesized predominantly late during infection ("late" proteins) as well as transcripts for "early" proteins (7, 17). Based on the analysis of the proteins synthesized in infected cells in the presence of low levels of actinomycin D, others have postulated that actinomycin D selectively inhibits the transcription of vRNA segments coding for late proteins (18). To assay for transcripts for early proteins, we used hybridization probes specific for segments 1 to 3, 5, and 8, which code for the P proteins, NP (nucleocapsid), and NS (nonstructural protein), respectively. To assay for transcripts for late proteins, we used a hybridization probe specific for segment 7, which codes for the M (membrane) protein. The nuclear cRNA synthesized in the presence of actinomycin D and CM contains transcripts for both early and late proteins (Table 5). The ratio of the amounts of the individual transcripts is similar, if not identical, to that observed in the cRNA found in the nucleus of cells treated with CM alone (Table 3). Thus, actinomycin D does not selectively inhibit the transcription of certain vRNA segments.

To establish whether the poly(A)-containing cRNA appearing in actinomycin D-treated cells at 2.5 h after infection was actually functional viral mRNA, its efficiency of translation by a micrococcal nuclease-treated L-cell extract was determined. The [³⁵S]methionine-labeled proteins synthesized were subjected to electrophoresis and visualized by autoradiography (Fig. 4). In the absence of added RNA, the L-cell extract synthesized a major protein migrating between the NP and M proteins and a minor protein migrating slightly lower than the viral P₁ protein. Cytoplasmic poly(A)⁺ RNA from infected, untreated cells stimulated the synthesis of all of the nonglycosylated virus-specific proteins (P₁, P_{2,3}, NP, M, NS) and possibly the nonglycosylated precursor to the HA glycoprotein (migrat-

FIG. 4. Polyacrylamide gel electrophoresis of proteins synthesized *in vitro* by an L-cell extract programmed with poly(A)-containing RNA from the nucleus and cytoplasm of infected cells. Poly(A)⁺ RNAs extracted from nuclear (NUC) and cytoplasmic (CYT) fractions of cells infected in the presence (+AD) or absence (-AD) of actinomycin D (2 µg/ml) at 2.5 h were translated in a micrococcal nuclease-treated L-cell extract. The [³⁵S]-methionine-labeled proteins were separated by electrophoresis as described in the text. ³⁵S-labeled virion proteins and infected cell proteins were used as markers (lanes 1 and 2, respectively). The following amounts of infected cell RNA were used to program *in vitro* translation: *in vivo*-synthesized viral cRNA, 1 µg; poly(A)⁺ cytoplasmic RNA from untreated cells, 1.8 µg; poly(A)⁺ cytoplasmic RNA from AD-treated cells, 7.2 µg; poly(A)⁺ nuclear RNA from untreated cells, 0.2 µg; poly(A)⁺ nuclear RNA from AD-treated cells, 0.1 µg. The distribution of RNA in the cell is approximately 90% cytoplasmic and 10% nuclear (29; data not shown).

ing between the P and NP proteins). Four times as much poly(A)-containing cytoplasmic RNA from infected, actinomycin D-treated cells stimulated the synthesis of the same proteins, but in lower amounts. Thus, even though this fraction, as judged by hybridization analysis, contained only 2% of the virus-specific cRNA of untreated cells (Table 4), it was functional in this translation assay. We conclude that the cytoplasm of actinomycin D-treated cells contains viral mRNA translatable into both early (NP and NS) and late (M) proteins. The poly(A)-containing nuclear RNA from infected cells, whether or not treated with actinomycin D, stimulated the *in vitro* synthesis of early and late virus-specific proteins. The similar amount of stimulation by these two nuclear RNAs is consistent with the approximate amounts of poly(A)⁺ cRNA detected by hybridization (Tables 2 and 4). How-

ever, in contrast to the distribution of cRNA between the nucleus and cytoplasm of actinomycin D-treated cells (Table 4), the amount of translatable viral mRNA obtained from the nucleus of these cells was not seven- to eightfold greater than the amount obtained from the cytoplasm. The reasons for the lower yield of functional viral mRNA from the nucleus are not at present known.

The effect of α -amanitin on virus-specific RNA synthesis. The inhibition of influenza virus replication by α -amanitin has been shown to be due to inhibition of the host cell RNA polymerase II (13, 28). When this drug is added at the beginning of infection, no detectable virus-specific proteins are synthesized (13, 28). Two previous reports (16, 28) indicated that this drug inhibited early RNA synthesis in infected cells, and one experiment in the literature suggested

that α -amanitin inhibited cRNA synthesis (26).

We were unable to detect significant viral transcription or vRNA replication when α -amanitin (20 μ g/ml) was added at the time of virus adsorption (Table 6).

When α -amanitin was added between 20 and 40 min after infection, a significant amount of viral cRNA synthesis occurred (Fig. 5). By 1.5 h, viral RNA transcription became completely insensitive to the addition of the drug. The sensitivity of vRNA synthesis to α -amanitin paralleled that of cRNA synthesis (results not shown). Actinomycin D and α -amanitin seem to inhibit virus-specific RNA synthesis by different mechanisms. First, no consistent evidence for nuclear accumulation of cRNA transcripts was observed in any of the α -amanitin-treated cultures. Second, the insensitivity of cRNA synthesis to α -amanitin addition at 1.5 h is in contrast to the results obtained with actinomycin D, where addition at 1.5 h inhibited further cRNA synthesis with little or no inhibition of vRNA replication (29; data not shown).

DISCUSSION

The results presented in this study point to the nucleus as the site of primary transcription of the influenza virus genome. The addition of cycloheximide at the beginning of infection inhibited vRNA replication and therefore presumably confined viral RNA transcription. Under these conditions, about 50% of the cRNA synthesized was found in the nucleus during the first 2 h after infection, and the nucleus always contained a larger fraction of the cRNA than in infected cells not treated with cycloheximide. The primary transcripts found in the nucleus (Tables 2 and 3) were shown to be polyadenylated transcripts of each of the genome vRNA segments. When actinomycin D was added at the beginning of infection, no detectable vRNA replication occurred, and the total amount of cRNA was reduced to 12% of that found in untreated cells. However, the absolute amount of cRNA molecules detected in the nucleus was

essentially not reduced. Consequently, in actinomycin D-treated cells, almost 90% of the cRNA was found in the nucleus. This nuclear cRNA was shown to be comprised of polyadenylated transcripts of all the vRNA segments for which we assayed, including segments coding for both early and late proteins (Table 5). Additionally, these cRNA molecules were shown to be functional viral mRNA's by in vitro translation experiments. These results disprove our earlier hypothesis that the cRNA synthesized in the presence of actinomycin D is somehow defective (29) and for this reason fails to migrate from the nucleus to the cytoplasm. A more likely explanation comes from recent experiments of

FIG. 5. Effect of the time of α -amanitin addition on the synthesis of virus-specific cRNA. Cells were infected, and, at various times from 1 h before adsorption ($t = -2$ h) to 1.5 h after infection, the medium was supplemented with 20 μ g of α -amanitin per ml. At 2.5 h the number of molecules of virus-specific RNA accumulated in the nucleus and cytoplasm was determined as described in the text. The results are expressed as the fraction of the total cRNA accumulated in the drug-treated cultures relative to the total cRNA accumulated in an untreated culture, at 2.5 h. Each symbol represents a separate experiment.

TABLE 6. Content of cRNA and vRNA in the nucleus and cytoplasm of untreated and α -amanitin-treated infected cultures^a

Treatment	Time drug added (h)	Time of collection (h)	Avg no. of viral genome equivalents in:					
			Nucleus		Cytoplasm		Whole cell	
			cRNA	vRNA	cRNA	vRNA	cRNA	vRNA
None		0	38	1,062	23	1,654	61	2,716
None		3	872	5,372	9,876	22,623	10,748	27,995
α -Amanitin	-2	3	5	865	34	1,676	39	2,541
α -Amanitin	-1	3	94	575	34	1,157	128	1,732

^a RNA extracted from untreated and α -amanitin (20 μ g/ml)-treated infected cultures was used to determine the average number of genome equivalents as described in the text.

Levis and Penman (14), who have reported that actinomycin D blocks the migration of newly synthesized cellular mRNA sequences from the nucleus to the cytoplasm. We propose that actinomycin D has the same effect on newly synthesized influenza viral mRNA, with the implicit assumption that primary transcription occurs in the nucleus. Definitive proof of the site of primary transcription might be obtained by studying pulse-labeled, rather than steady-state, cRNA, and such experiments are in progress.

Parenthetically, our results indicate that actinomycin D does not selectively inhibit the transcription of the vRNA segment coding for the late protein, the M protein. This argues against the hypothesis of Minor and Dimmock (18) that this drug acts directly at the transcriptional level to inhibit the transcription of segments coding for late proteins. Their hypothesis was based on the observation that, in cells treated with levels of actinomycin D lower than that employed in our study, the synthesis of late proteins was inhibited more than that of early proteins. This observation can be better explained by other known effects of actinomycin D. For example, because actinomycin D blocks the migration of cRNA from the nucleus to the cytoplasm, it is conceivable that at low concentrations of this drug some transcripts might be selectively retained in the nucleus. Also, because vRNA replication is inhibited in actinomycin D-treated cells, it might be expected that the switch from primary to amplified transcription would also be inhibited.

Our experiments with α -amanitin suggest that an RNA product of the host nuclear RNA polymerase II is required for the initiation of influenza viral RNA transcription. The required RNA product of this host enzyme can be presumed to be one or more host mRNA's and/or their precursors, as Bouloy et al. (1) have recently demonstrated that globin mRNA's and also other eucaryotic mRNA's are effective primers for influenza viral RNA transcription in vitro catalyzed by the virion-associated transcriptase. Thus, the observed in vivo early sensitivity and the late (1.5 h) refractoriness of cRNA synthesis can be explained by the availability of a host-coded mRNA primer. At the time of infection, the amount of this primer mRNA in the cell would be insufficient for viral cRNA synthesis, so that de novo synthesis of primer mRNA molecules would be required. By 1.5 h after infection, a sufficient amount of primer mRNA molecules apparently has accumulated in the infected cell so that their further synthesis would be unnecessary. The early effect of actinomycin D would be analogous to that of

α -amanitin, except that the inhibition of host mRNA synthesis is not complete and some viral cRNA is synthesized, although these molecules are restricted to the nucleus. The late effect of actinomycin D on amplified cRNA synthesis could be explained by the ability of this drug to block RNA migration from the nucleus to the cytoplasm. In this case it would be blocking the migration of primer mRNA molecules, rather than of cRNA molecules, as the late addition of actinomycin D does not cause a detectable accumulation of cRNA molecules in the nucleus (29; unpublished data). According to this hypothesis, amplified transcription occurs in the cytoplasm, which is consistent with the observed intracellular distribution of cRNA molecules after 1.5 h of infection (Table 1) and with the pulse-labeling data of Hay et al. (6).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-11772 from the National Institute of Allergy and Infectious Diseases and CA-08748 from the National Cancer Institute, by National Science Foundation grant PCM 76-81525, by American Cancer Society grant VC-155C, and by an appropriation from the Commonwealth of Pennsylvania. The PDP-11/70 computer facility used in this study was developed through Public Health Service grants CA-06927 and CA-22780 from the National Cancer Institute.

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