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NOTES

Influenza Virus RNAs in the Cytoplasm of Chicken Embryo Cells Treated with 3'-Deoxyadenosine

CARROLL L. PRIDGEN

Laboratories of Virology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101

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3'-Deoxyadenosine (75 to 100 μg/ml) permitted analysis of the cytoplasmic influenza virus-specific RNAs synthesized early in the replicative cycle—a phase that has hitherto been obscured by host cell RNA synthesis. In addition, late in the cycle (6 to 8 h) complementary virus-specific RNAs were the predominantly labeled species, suggesting that higher concentrations of 3'-deoxyadenosine selectively inhibit influenza viral genome replication.

The study of influenza virus-specific RNA metabolism in the cell had been hampered by the lack of methods to reduce host cell RNA synthesis without inhibiting transcription of viral complementary RNAs (13, 16). However, it has now been shown that up to 50 μg of 3'-deoxyadenosine (3'-dA) per ml does not inhibit influenza virus replication, transcription, or adenylation (5, 10), and this offers a method for uncovering virus-specific RNAs synthesized in cells.

In this study, 3'-dA (75 to 100 μg/ml) was used to inhibit host cell RNA synthesis in order that the sedimentation properties and amounts of Turkey/Ontario/7732 influenza virus RNAs in the cytoplasm of chicken embryo cells could be analyzed at any time in the replicative cycle. To analyze the RNAs synthesized in the presence of 3'-dA throughout the replication cycle, RNAs were biosynthetically labeled in the presence of 3'-dA at several time periods. At 6 h (Fig. 1) and 8 h (data not shown) after infection, the distribution of RNAs from infected cells treated with 3'-dA (Fig. 1A) or actinomycin D (Act-D) (Fig. 1B) was similar. Both samples contained RNAs that sedimented predominantly at 18S, in agreement with previously published data on RNA synthesized in the presence of Act-D (4). Six times more label was present in the 18S region of the gradient in the infected cell cytoplasm than in the uninfected cell control (Fig. 1A). At 3 h, however, the RNAs from infected cells treated with Act-D sedimented at 8 and 12S, with little 18S RNA present (Fig. 2B), whereas RNAs isolated from 3'-dA-treated cells sedimented primarily at 18S, with additional peaks at 8 and 14S (Fig. 2A). In addition, the amount of radioactivity incorporated in the presence of 3'-dA was about one-half the amount incorporated at the 6- or 8-h time period. The sedimentation properties of the single-stranded RNAs from 3'-dA-treated cells were similar to the sedimentation patterns found at later times in infected cells treated with either drug. However, the observation that the virus-specific RNAs in the 8 and 14S RNA peaks (Fig. 2) were more evident early suggested that some RNAs were synthesized in different proportions at 3 h than at 6 or 8 h, which is in agreement with previous data (1).

To analyze the sedimentation properties of the RNase-resistant structures in Turkey/Ontario/7732-infected cells, a portion of each fraction of the gradients (Fig. 1 and 2) was treated with RNase A. Except for the RNAs isolated from the cytoplasm of Act-D-treated cells early in infection (Fig. 2B), all preparations contained an RNase-resistant structure sedimenting at about 14S (Fig. 1A, B, and 2A). However, at 3 h (Fig. 2A) there was a greater proportion of RNase-resistant radioactivity sedimenting slower (6S) than at 6 h (Fig. 1A) after infection. Although there was no RNase resistance found at 14S in uninfected cell RNA, there was some RNase-resistant material that sedimented at approximately 6S in gradients containing uninfected cell RNA (data not shown), casting doubt as to the viral origin of the smaller structure. The sedimentation values of the 14S RNase-resistant structures correlate with the value previously shown for structures proposed to be intermediate in influenza virus RNA synthesis (12).

To determine the amount of complementary

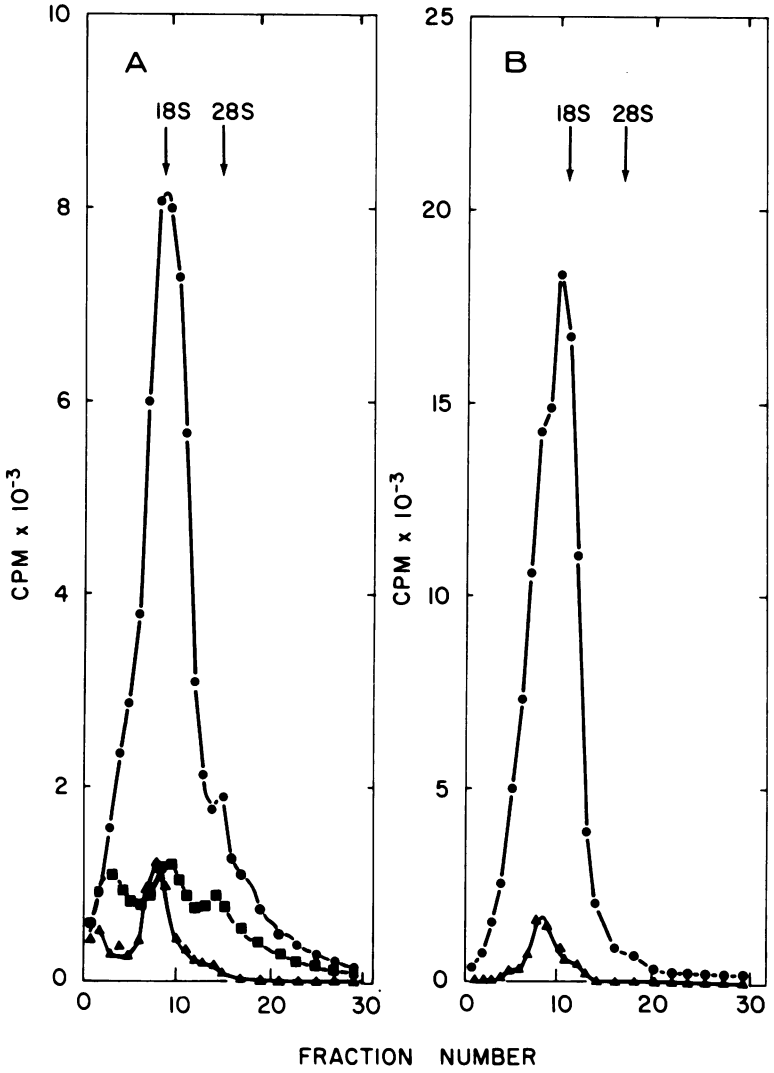

FIG. 1. *Cytoplasmic RNAs from influenza virus-infected cells at 6 h after infection. Chicken embryo cell cultures (3) were infected with one PFU of a cloned isolate of Turkey/Ontario/7732/66 (Hav5) strain of influenza A per cell (17). The infected cultures were maintained in minimal essential medium with 4% bovine serum albumin fraction V at 37 C in 5% CO_2. Infected cells were treated at 4 h after infection with either 3'-dA (75 μg/ml, Sigma Chemical Co.) (A) or Act-D (5 μg/ml, Merck & Co., Inc.) (B) for 1 h and were labeled in the presence of the drug with [^{3}H]uridine (50 μCi/ml; specific activity, 27 to 29 Ci/mmol) for an additional hour. Uninfected chicken embryo cells treated with 3'-dA served as a control (■). At 6 h, cytoplasmic extracts were prepared (11), and the RNAs were extracted from the cytoplasm as described previously (14). The gradients (containing 15 to 30% sucrose acetate-buffered saline, 0.01 M EDTA, and 0.5% sodium dodecyl sulfate) were centrifuged in an SW-27 rotor at 20 C for 16.5 h at 20,000 rpm. A portion (10%) of each gradient fraction was assayed for total acid-precipitable counts (●) and RNase resistance (▲), and radioactivity was determined as previously described (14). The remainder was used for analysis by hybridization (Experiment 1, Table 1). Absorption profile of ribosomal RNAs provided internal markers for 18 and 28S* RNA.

RNA synthesized in the presence of 3'-dA, portions of each gradient (Fig. 1 and 2) and similar fractions from other experiments containing 8-h samples were combined and annealed with unlabeled genomic RNA (Table 1). At 3 h after infection (Fig. 2), 61% of the labeled RNA in the cytoplasm of 3'-dA-treated cells was hybridized, whereas, at 6 h (Fig. 1), 71% of the labeled RNA was virus-specific (Experiment 1, Table 1). On the other hand, RNAs synthesized in the presence of Act-D contained a lower proportion of complementary RNA at 3 h (39%) and 6 h (29%)

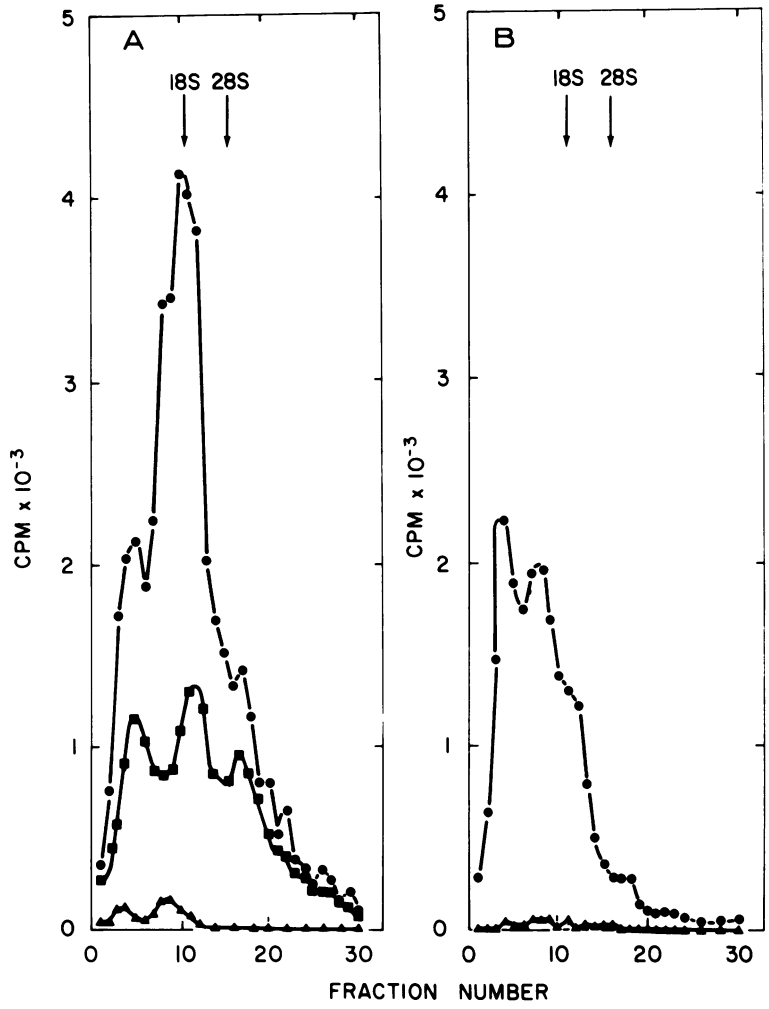

FIG. 2. *Cytoplasmic RNAs from influenza virus-infected cells at 3 h after infection. Infected cells were treated with either 3'-dA or Act-D at 1 h after infection, and the RNAs were labeled, extracted and processed as described in the legend to Fig. 1.*

(Experiment 1, Table 1). The high self-annealing values of the samples suggested that both strands were present in all preparations. It was not known, however, how much radioactive label was contributed by each strand in the 3'-dA-treated cell RNAs. In the preparations from Act-D-treated cells, virion RNA may have been the predominantly labeled strand in the self-annealed preparations since Act-D inhibited complementary RNA synthesis (Experiment 1, Table 1). Experiments 2 and 3 (Table 1) showed that, by 6 h after infection, complementary RNA was the major labeled RNA species in the cytoplasm of infected 3'-dA-treated cells. In addition, the slower-sedimenting RNAs (2-10S) in Experiment 2 were shown to contain virus-specific complementary RNA, although not always in the same proportions as the faster-sedimenting RNAs (Table 1). The values presented in Table 1 are representative only of the proportion of the total labeled RNA in the cytoplasm of infected, 3'-dA-treated cells that hybridizes to virion RNA. Without a complementary RNA probe, it was impossible to determine what proportion of the remaining nonannealed RNA was virion RNA. As shown by others (15), these data also showed that the machinery needed for transcription was present 8 h after infection. In addition, the data (Table 1) showed that complementary RNA was the majority species of labeled RNA in the cytoplasm of infected cells treated with 3'-dA at 6 or 8 h after infection. These data differed from the results of previous studies (2, 8, 16). The observed differences could be explained in the following ways. (i) Some of the previous analyses were made in the pres-

TABLE 1. *RNAs present in cytoplasm of 3'-dA-treated cells*

Time after infection (h)	Drug	S values[a]	% Unannealed RNase resistance	Radioactivity		% Self annealed[b]	Radioactivity		% Hybridized[b]
				Total counts/min	RNase counts/min		Total counts/min	RNase counts/min	
Expt 1[c]									
3	3'-dA		15	445	97	22	444	270	61
	Act-D		15	396	158	40	455	173	38
		8–20							
6	3'-dA		14	408	173	42	418	296	71
	Act-D		9	412	175	43	443	129	29
Expt 2[d]									
3	3'-dA	2–10	1	ND[e]	ND	ND	263	84	32
		12–20	5	305	9	3	448	144	32
6	3'-dA	2–10	2	ND	ND	ND	191	93	49
		12–20	5	423	65	15	390	232	60
8	3'-dA	2–10	6	ND	ND	ND	149	76	51
		12–20	11	429	59	14	406	278	69
Expt 3									
6	3'-dA		5	540	90	17	585	354	61
	Act-D		5	488	109	22	470	102	22
		8–20							
8	3'-dA		6	490	156	32	524	434	83
	Act-D		6	427	134	31	415	137	33

[a] Sedimentation values of RNAs in fractions pooled from gradients for analysis by hybridization, using the technique of Kingsbury (6).

[b] These values are averages of at least two separate hybridizations of the same material.

[c] RNA preparations were lyophilized before the annealing buffer (25 μl) was added. Saturating amounts (20 μg/ml) of virion RNA extracted (7) from purified Turkey/Ontario/7732 (9) were used.

[d] Experiments 2 and 3 were annealed in 100 μl of buffer without prior lyophilization to reduce self annealing. Annealing was done with saturating amounts of virion RNA (2.5 μg/ml) for 2 h at 80 C.

[e] ND, Not determined.

ence of Act-D, a known inhibitor of influenza virus-specific transcription (2, 12). (ii) Other analyses of RNA from influenza virus-infected cells without Act-D were made on RNAs from total cell extracts, which contain both nuclear and cytoplasmic RNAs (12, 16), or from ribonucleoprotein structures (8). None of these studies analyzed all of the RNAs contained in just the cytoplasm. (iii) Another possibility is that virion RNA was selectively lost during the extraction procedure used in this study. (iv) Finally and most likely, higher concentrations of 3'-dA may inhibit replication, either by specifically inhibiting the enzyme synthesizing virion RNA or by some secondary effect on protein synthesis, as shown with Newcastle disease virus and Sendai virus (S. R. Weiss and M. A. Bratt, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, 5227, p. 251; C. Pridgen and D. W. Kingsbury, unpublished data).

Although 3'-dA may have inhibited replication of virion RNA, transcription of virus-specific RNAs continued in the presence of 3'-dA. This study showed that 3'-dA was an adequate tool to aid in the analysis of virus-specific transcripts in the cytoplasm of cells infected with influenza virus.

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LITERATURE CITED

1. Avery, R. J., and N. J. Dimmock. 1975. Temporal control of transcription of influenza virus RNA. Virology 64:409–414.
2. Content, J., and P. H. Duesberg. 1971. Base sequence differences among the ribonucleic acids of influenza virus. J. Mol. Biol. 62:273–285.
3. Darlington, R. W., A. Portner, and D. W. Kingsbury. 1970. Sendai virus replication: an ultrastructural comparison of productive and abortive infections in avian cells. J. Gen. Virol. 9:169–177.
4. Duesberg, P. H. 1968. The RNAs of influenza virus. Proc. Natl. Acad. Sci. U.S.A. 59:930–937.
5. Etkind, P. R., and R. M. Krug. 1974. Influenza viral messenger RNA. Virology 62:38–45.
6. Kingsbury, D. W. 1966. Newcastle disease virus RNA. II. Preferential synthesis of RNA complementary to

parental viral RNA by chick embryo cells. J. Mol. Biol. 18:204–214.
7. Kingsbury, D. W., and R. G. Webster. 1969. Some properties of influenza virus nucleocapsids. J. Virol. 4:219–225.
8. Krug, R. M. 1972. Cytoplasmic and nucleoplasmic viral RNPs in influenza virus-infected MDCK cells. Virology 50:103–113.
9. Laver, W. G. 1969. Purification of influenza virus, p. 82–86. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
10. Mahy, B. W. J., N. J. Cox, S. J. Armstrong, and R. D. Barry. 1973. Multiplication of influenza virus in the presence of cordycepin, an inhibitor of cellular RNA synthesis. Nature (London) New Biol. 243:172–174.
11. Milcarek, C., R. Price, and S. Penman. 1974. The metabolism of poly (A) minus mRNA fraction in HeLa cells. Cell 3:1–10.
12. Nayak, D. P., and M. A. Baluda. 1968. An intermediate in the replication of influenza virus RNA. Proc. Natl. Acad. Sci. U.S.A. 59:184–191.
13. Pons, M. W. 1973. The inhibition of influenza virus RNA synthesis by actinomycin D and cycloheximide. Virology 51:120–128.
14. Pridgen, C., and D. W. Kingsbury. 1972. Adenylate-rich sequences in Sendai virus transcripts from infected cells. J. Virol. 10:314–317.
15. Repik, P., A. Flamand, and D. H. L. Bishop. 1974. Effect of interferon upon the primary and secondary transcription of vesicular stomatitis and influenza viruses. J. Virol. 14:1169–1178.
16. Scholtissek, C., and R. Rott. 1970. Synthesis *in vivo* of influenza virus plus and minus strand RNA and its preferential inhibition by antibiotics. Virology 40:989–996.
17. Webster, R. G., C. H. Campbell, and A. Granoff. 1971. The *in vivo* production of new influenza A viruses. I. Genetic recombination between avian and mammalian influenza viruses. Virology 44:317–328.