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Use of Specific Radioactive Probes to Study Transcription and Replication of the Influenza Virus Genome

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Specific radioactive probes have been obtained for both influenza virion RNA (vRNA) and for its complement (complementary RNA or cRNA): ^{32}P-labeled complementary DNA (cDNA) synthesized with the avian sarcoma virus reverse transcriptase, and [^{125}I]vRNA, respectively. From the kinetics of annealing of these two probes to RNA from canine kidney cells infected with the WSN strain of influenza virus, we have determined the average number of cRNA and vRNA sequences in the nucleus and cytoplasm as a function of time after infection. Immediately after infection, a small amount of vRNA is detected, presumably from the inoculum virus. As expected, the amount of cRNA is insignificant. During the first 1.75 h of infection, the most significant increase observed is in cRNA sequences. Most of these cRNA sequences are found in the cytoplasm, but a significant amount (30%) is found in the nucleus. During this time, a small but significant increase in vRNA is also detected in the nucleus and cytoplasm. From 1.75 to 2.75 h, the absolute amounts of both cRNA and vRNA increase, predominantly in the cytoplasm, with cRNA remaining as the majority species. Subsequently, the amount of vRNA increases with respect to cRNA and becomes the majority species. At 3.75 h, 95% of both cRNA and vRNA are found in the cytoplasm. Addition of actinomycin D at 1.75 h completely suppresses the subsequent ninefold increase in cRNA and does not have a significant effect on the subsequent 14-fold increase in cytoplasmic vRNA. This assay is also able to detect the cRNA produced as a result of primary transcription, operationally defined as the cRNA produced in the presence of 100 μg of cycloheximide per ml added at zero time of infection. Increases in cRNA in the presence of cycloheximide are detectable in both the nucleus and the cytoplasm. Addition of actinomycin D as well as cycloheximide at zero time completely suppresses the appearance of cRNA in the cytoplasm, whereas a large fraction (50%) of the increase in nuclear cRNA still occurs.

Previous studies have been undertaken to characterize the transcription and replication of the influenza virus genome in infected cells (3, 30, 33). A major problem in such studies has been that virus-specific RNA represents only a small fraction of the total RNA in infected cells and that inhibitors like actinomycin D cannot be used to suppress host cell RNA synthesis because these drugs also inhibit influenza virus replication (2, 18, 29, 32, 40). A similar problem exists for RNA tumor viruses. With these viruses, as Garapin et al. (14) first demonstrated, a solution has been to measure the kinetics of hybridization of RNA from infected cells to specific radioactive nucleic acid probes. From an analysis of such data the number of molecules of viral RNA can be deduced (26). Moreover, the assay has the sensitivity to detect as little as one molecule per cell (4). The application of such a technique to influenza virus replication is complicated by the fact that both the virion RNA (vRNA) and its complement (cRNA) are present in the infected cells. Consequently, two specific probes are necessary. As a probe for cRNA we used vRNA labeled in vitro with ^{125}I. As a probe for vRNA we synthesized ^{32}P-labeleld DNA from a vRNA template using purified reverse transcriptase. This transcription was facilitated by the presence during synthesis of primer oligonucleotides derived from calf thymus DNA by limit digestion with deoxyribonuclease. As previously shown these primers allow efficient copying of RNAs like influenza vRNA (37a) which lack polyadenylate sequences. From an analysis of the kinetics of annealing of these two radioactive probes to RNA fractions from cells infected by influenza virus, we have been

able to determine the number of copies of both cRNA and vRNA in the nucleus and cytoplasm at various times after infection. We have quantitated the effect of actinomycin D on the appearance of these molecules. In addition, because of the sensitivity of the hybrization technique, we have been able to study primary transcription, operationally defined as the transcription occurring in the presence of the protein synthesis inhibition cycloheximide (CM) added at the time of infection (3). The effect of actinomycin on this transcription has also been examined.

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 (21, 22).

Preparation of infected cell cytoplasmic and nuclear RNA. Monolayer cultures of MDCK cells grown on roller bottles were infected with WSN virus at a multiplicity of 30 to 60 plaque-forming units/cell. Adsorption was for 1 h at 4°C. After adsorption, the cells were washed two times to remove inoculum virus. Growth medium (reinforced Eagle medium containing 2% calf serum) prewarmed to 37°C was added, and the cells were incubated at 37°C. Zero time corresponds to the time at which the cells were brought to 37°C. Where indicated, CM at 100 μg/ml and/or actinomycin D at 2 μg/ml was added. One hour before collection of the cells, [^{3}H]adenosine at 5 μCi/ml was added to label the infected-cell RNA. The radioactivity in the infected-cell RNA enabled us to monitor the steps during the preparation of this RNA for annealing.

At the indicated times, the infected cells were collected into reticulocyte standard buffer (RSB) (0.01 M KCl, 0.0015 M $MgCl_2$, 0.01 M Tris-hydrochloride, pH 7.4) and were ruptured with 25 strokes in a Dounce homogenizer. The cell extract was fractionated into nucleus and cytoplasm as described previously (24), which yields a nuclear preparation free of detectable cytoplasmic contamination as monitored by the absence of 18S rRNA (24, 28). After centrifugation of the cell extract for 8 min at 250 × *g*, the pelleted crude nuclei were washed, first with RSB, and then with RSB containing a mixture of Tween-40 and deoxycholate, to remove residual cytoplasmic material. The original supernatant, and the two washes of the nuclei were combined to give the cytoplasmic extract. The detergent-washed nuclei were taken up in high-salt buffer (0.05 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris-hydrochloride, pH 7.4) containing 80 μg of electrophoretically pure deoxyribonuclease per ml and were pipetted up and down until the viscosity disappeared.

The cytoplasmic and nuclear extracts were made 0.02 M in EDTA, 2% in sodium dodecyl sulfate and 0.1 M in Tris-hydrochloride, pH 9.0, and were extracted three times with phenol-chloroform at pH 9.0. Phenol was removed by three ether extractions and the ether was removed by bubbling N_2 through the solution. The RNA was desalted by chromatography through Sephadex G-50 in water. The RNA in the excluded volume (monitored by its ^{3}H-labeled radioactivity) was pooled and lyophilized to dryness.

Preparation of purified viral cRNA. The procedures for obtaining from infected cells a preparation of viral cRNA in which all of the radiolabel derived from a [^{3}H]adenosine or a [^{3}H]uridine precursor is in viral cRNA has been described previously (10).

Determination of the RNA content of MDCK cells. Monolayer cultures of MDCK cells were collected by trypsinization. The cells were counted. The total nucleic acids were extracted with phenol-chloroform, and the amount of RNA was determined by the orcinol reaction to be 25×10^{-12} g of RNA per MDCK cell. Another set of monolayer cultures were collected by scraping, and the nuclear and cytoplasmic RNA was obtained as described above. The amount of RNA in these two fractions was determined by both absorbancy at 260 nm and the orcinol reaction. Both methods gave the same results. The average of several experiments indicated that 9% of the RNA is nuclear, and 91% is cytoplasmic.

^{32}P-labeled DNA probe for vRNA. RNA was extracted from purified virions (22) and added at a concentration of 2 μg/ml to 0.2 ml of a reaction mixture containing 0.1 M Tris-hydrochloride, pH 8.1, 0.01 M $MgCl_2$, 2% mercaptoethanol; dGTP, dTTP, and dCTP at 10^{-4} M; 10^{-6} M [α-^{32}P]dATP (110 Ci/mM, New England Nuclear); 500 μg of degraded calf thymus DNA primers/ml; 0.2 U of avian sarcoma virus polymerase/ml; 33 μg of actinomycin D/ml (Calbiochem). The presence of actinomycin selectively inhibits the synthesis of double-stranded DNA (15). The preparation and use of the degraded calf thymus DNA primers has been described elsewhere (Taylor et al. Biochim. Biophys. Acta, in press). These primers increase the efficiency of transcription of influenza vRNA by about 300-fold. The avian sarcoma virus polymerase was purified from the B77 strain as previously described (11). After synthesis for 3 h at 37°C, the DNA product was extracted with phenol, passed through a G-50 Sephadex column (40 by 0.9 cm), treated with 0.6 N NaOH for 1 h at 37°C to hydrolyze RNA, and then collected by ethanol precipitation. The final product had a modal sedimentation value of 5S and a specific activity of about 100×10^6 cpm/μg.

^{125}I-labeled RNA probe for cRNA. One microgram of RNA extracted from purified virions was labeled in the presence of 1 mCi of ^{125}I (New England Nuclear) by the thallium chloride procedure of Tereba and McCarthy (38). After passage through a G-50 Sephadex column, the labeled RNA was collected by ethanol precipitation. This RNA had a modal sedimentation value of about 5S and a specific activity of at least 30×10^6 cpm/μg.

Kinetics of annealing of labeled probes. The RNA sample to be assayed was resuspended in water and heated at 95°C for 3 min. It was then mixed with about 10,000 cpm of both [^{125}I]vRNA and [^{32}P]cDNA and equilibrated at 68°C. In the analysis of nuclear and cytoplasmic RNAs, about 100 and 1,000 μg, respectively, were used per annealing mixture. Relative to the labeled probes, these values correspond to mass excesses of about 10^6 and 10^7, respectively. To begin an annealing, a concentrated salt

solution prewarmed to 68°C was added to bring the annealing mixture to 0.6 M NaCl, 0.04 M Tris-hydrochloride, pH 7.4, 0.002 M EDTA. This mixture (150 μl) was overlayed with prewarmed mineral oil (50 μl) and incubated at 68°C in a 2-ml conical tube. After 0.61, 2.5, 10, 40, 160, and 1,200 min, portions of 20 μl were removed, chilled, and stored at −20°C until the nuclease assays could be performed. To perform the latter assays, each portion was diluted with 0.3 M NaCl and divided into six equal samples. To assay the annealing of [^{125}I]vRNA, two samples were digested with 20 μg of boiled pancreatic RNase per ml in 0.3 M NaCl for 1 h at 37°C. To assay the annealing of [^{32}P]cDNA, two samples were digested with 1,200 U/ml of the single-stranded specific S1 nuclease (Miles Laboratories) for 1 h at 37° (26). The remaining two samples were incubated in the absence of enzymes. After incubation, 80 μg of carrier DNA was added and the samples were precipitated with 10% trichloroacetic acid and collected onto glass fiber filters. ^{125}I was assayed in a Beckman gamma counter and ^{32}P was assayed by Cerenkov radiation in a Beckman LS-233 scintillation counter. In these two counting procedures the ^{3}H present in the infected-cell RNA is not detected. All samples were counted for 10 min and the values presented represent the average of duplicate assays.

RESULTS

Characterization of probes for cRNA and vRNA. Our assay is based upon the ability of viral sequences present in infected-cell RNA to anneal to a labeled complementary nucleic acid probe, thereby protecting the latter against a subsequent nuclease digestion. To assay for cRNA sequences we have used [^{125}I]vRNA and measured the resistance to digestion with pancreatic RNase. To assay for vRNA sequences we have used [^{32}P]cDNA transcribed from vRNA with reverse transcriptase. The extent of annealing of this probe was measured with the single-stranded specific S1 nuclease. To determine whether these two probes had the desired specificities, the following control annealing studies were performed.

The first control was to measure the kinetics of annealing of the labeled probes in the presence of excess vRNA, as extracted from purified virions. As shown in Fig. 1a, ultimately 100% protection was conferred upon the [^{32}P]cDNA probe, whereas the [^{125}I]vRNA remained unprotected. The initial nuclease resistance of both probes was about 5%. In data other than that shown in Fig. 1, this background has been subtracted from the data.

The second control was to measure the annealing in the presence of excess cRNA. The latter cRNA (labeled with [^{3}H]uridine) was obtained from infected cells as described previously (10). This preparation is isotopically pure cRNA. It is not chemically pure in that unlabeled host is probably present. Nevertheless, the amount of vRNA present, labeled or unlabeled, is insignificant with respect to the cRNA (10). The kinetics of annealing of the two labeled probes in the presence of the cRNA are shown in Fig. 1b. The [^{32}P]cDNA remained unprotected, whereas the extent of annealing of the [^{125}I]vRNA was ultimately 75 to 80%. We do not know why this extent is less than 100%. It is probably not a consequence of different viral sequences being present at different frequencies in the cRNA preparation because neither larger amounts of this RNA nor of any of the infected cell RNAs (to be described below) were able to increase the extent of annealing. Consequently, in data other than that shown in Fig. 1 the final extent of annealing has been normalized to 100%.

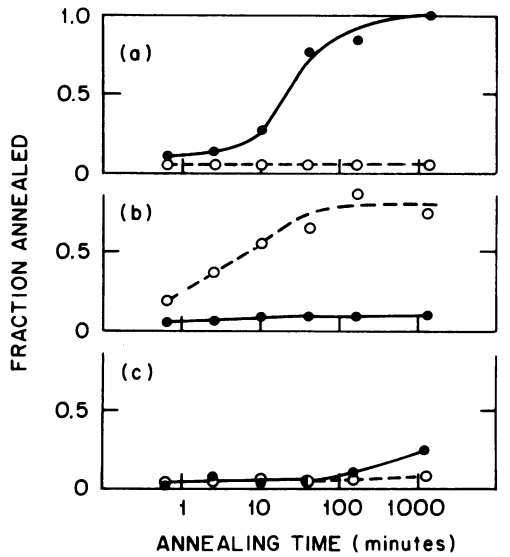

FIG. 1. *Time-dependent annealing of the labeled probes. The ^{32}P-labeled cDNA (●) and the ^{125}I-labeled vRNA (○) were submitted to annealing conditions for the times indicated in the presence of: (a) 0.4 μg of unlabeled vRNA per ml from purified virions; (b) a RNA fraction enriched for cRNA and free of detectable vRNA (as described under Materials and Methods); (c) no added RNA. The vertical axis shows the fraction of each labeled probe that was resistant to nuclease after the indicated annealing time. Further details are provided in the text.*

The third control was to test for possible annealing of the labeled probes to each other in the absence of added RNA (Fig. 1c). With the concentration of probes used and with annealing times not exceeding 200 min, such annealing is insignificant. A small but significant amount of annealing of [^{32}P]cDNA occurs by 1,000 min. Even this amount of annealing

would be competed out by the presence of an excess of unlabeled vRNA or cRNA. The addition to an annealing mixture of 1 mg of RNA per ml from uninfected cells does not significantly augment the amount of annealing observed in Fig. 1c. This shows that the two probes do not anneal to sequences present in uninfected cells.

The three control annealings described above were performed concurrently with each of the annealing experiments to be described subsequently.

Theoretical analysis of the annealing kinetics. Experimentally we can measure the kinetics of annealing of the labeled probes for cRNA and vRNA in the presence of an excess of RNA extracted from infected cells. We have sought to deduce from such data, first, the ratio of cRNA-to-vRNA sequences and, secondly, the absolute numbers of sequences of each per average nucleus or cytoplasm. To obtain this information the following theoretical analysis was made.

Consider the situation in which a sample of RNA from an infected cell is thoroughly denatured and then incubated under annealing conditions at a concentration C_r. At time $t = 0$, before any annealing, the concentration of free cRNA and vRNA in this RNA are P_0 and M_0, respectively. At any subsequent time the corresponding concentrations that remain unannealed are P and M where:

$$\frac{dP}{dt} = \frac{dM}{dt} = -kMP \qquad (1)$$

and k is a positive constant. Two important assumptions are implicit in the application of these equations to the experimentally measured annealing kinetics. They are, first, that during infection the influenza virus genome is transcribed uniformly, and, second, that the rate constant k, for the annealing between unlabeled cRNA and vRNA sequences is the same as that between the corresponding labeled and unlabeled sequences. These assumptions are considered in the Discussion.

The equation in (1) can be solved. For the case where M_0 is greater than P_0, the fractions of the vRNA and cRNA that have annealed at a given time are:

$$\frac{M_0 - M}{M_0} = \frac{1 - \exp[-k(M_0 - P_0)t]}{M_0/P_0 - \exp[-k(M_0 - P_0)t]}; \qquad \frac{P_0 - P}{P_0} = \frac{M_0}{P_0}\frac{M_0 - M}{M_0} \qquad (2)$$

At large values of the product $C_r t$, these equations asymptote to

$$\frac{M_0 - M}{M_0} \to \frac{P_0}{M_0}; \frac{P_0 - P}{P_0} \to 1 \qquad (3)$$

That is, in this case, where M_0 is greater than P_0, ultimately all of the cRNA will be annealed, whereas only that fraction P_0/M_0 of the vRNA will be annealed. Moreover, it can be deduced from the equations (2), that this fraction, P_0/M_0, is the ratio of the extent of annealing of vRNA to the extent of annealing of cRNA for all values of $C_r t$. The equations have analogous solutions for the alternative case of P_0 being greater than M_0.

The shape of the two curves obtained from the equations in (2) is dependent upon the value of P_0/M_0. Thus, when the experimentally determined annealing data for a given RNA sample are fitted to equations of the form in (2) by a least-squares method the value of P_0/M_0 is obtained.

To obtain the absolute values of P_0 and M_0 in equation (2) it is first necessary to determine the constant k. This can be done from the data shown in Fig. 1a, where annealing is carried out in the presence of excess pure vRNA (that is, $C_r \simeq M_o$ and $P_0 \ll M_0$). The value of the product $C_r t$ at which half of the labeled probe is annealed is known as the $C_r t_{\frac{1}{2}}$, and this value corresponds to $\ln 2/k$. The average value of $C_r t_{\frac{1}{2}}$ from seven experiments, after correction to standard salt conditions (6), is 0.022 ± 0.005 mol-s/liter. This yields the value of k as 35 ± 7 liters/mol-s.

Using the deduced values of P_0 and M_0 for an RNA sample from infected cells at concentration C_r, we can calculate the average number of genome equivalents of cRNA and vRNA. The additional information needed for this calculation is: the total molecular weight of the viral genome (5.9×10^6 d [23a, 31]). The mass of RNA per MDCK cell (25×10^{-12} g), the fraction of cellular RNA in the nucleus (9%), and Avogadro's number (6.02×10^{23} molecules/g-mol). For example, if a nuclear RNA preparation is at a concentration C_r and we have deduced the concentration of cRNA (P_0), then the average number of molecules of cRNA sequences present per nucleus is:

$$(P_0/C_r) \times (0.09) \times (25 \times 10^{-12}) \times (6.02 \times 10^{23}) \div (5.9 \times 10^6).$$

Detection of vRNA and cRNA in the nucleus and cytoplasm at various times after infection. Replicate cultures of MDCK cells were infected with influenza virus and subsequently incubated at 37°C for 0, 1.75, 2.75 or 3.75 h. From these cultures the nuclear and cytoplasmic RNAs were isolated. The kinetics of annealing of the two labeled probes in the presence of each RNA were measured. The results are shown in Fig. 2 as a function of the product $C_r t$, where C_r (moles/liter) is the con-

centration of the RNA and t (seconds) is the time of annealing. The extent of hybridization of the [^{125}I]vRNA and [^{32}P]cDNA is shown by the open and closed symbols, respectively. To obtain the respective amounts of cRNA and vRNA in each RNA fraction, the annealing data were subjected to the theoretical analysis described earlier. Theoretical curves of the form in equation (2) were fitted to the data by a least-squares method. The superimposed cross-hatching represents the computed curves with a range of one standard deviation. As described in the previous section, the parameters of the fitted curves allow the deduction of the ratio of cRNA/vRNA, as well as the absolute amounts of vRNA and cRNA. The latter are expressed as the average number of genome equivalents per nucleus or cytoplasm and from the sum of these values, the genome equivalents per cell (Table 1, experiment 1). The standard deviations shown in Fig. 2 and Table 1 contain two contributions: one from the experimental data itself and the other from the appropriateness of the theoretical formulation. In some cases the latter introduces a relatively large component (e.g., Fig. 2h) (see Discussion).

First, consider the total number of genome equivalents of cRNA and vRNA per cell. At zero time, the amount of cRNA is insignificant, and that vRNA which is detected is presumably derived from the inoculum virus. During the first 1.75 h, both cRNA and vRNA increase in amount, but the greater increase is for cRNA so that it becomes the majority species. During the period of 1.75 to 2.75 h, the absolute amounts of both cRNA and vRNA increase, with cRNA remaining the majority species. Beyond 2.75 h, the amount of vRNA increases with respect to cRNA and becomes the majority species. This occurs because of an increase in vRNA sequences and also in some experiments (see Table 1), because of an apparent decrease in cRNA sequences. This de-

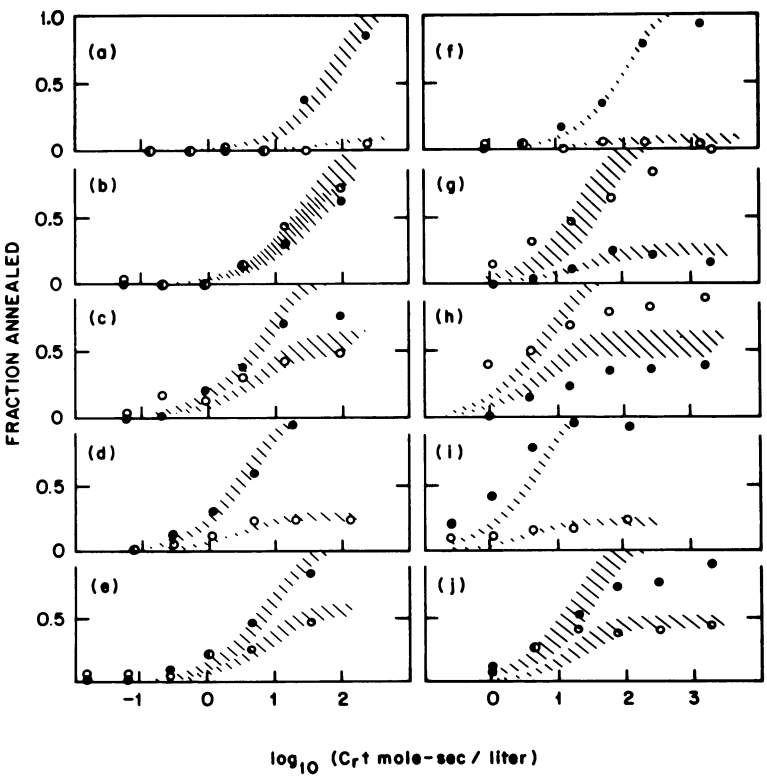

FIG. 2. *Annealing of labeled probes to RNA extracted from the nucleus and cytoplasm of MDCK cells at various times after infection. The [^{32}P]cDNA (●) and [^{125}I]vRNA (○) were annealed in the presence of either nuclear RNA, (a-e), or cytoplasmic RNA, (f-j). After the indicated values of C_0t, samples were removed and the fraction of the probes annealed was assayed by nuclease resistance. The infected cultures used were incubated at 37°C as indicated: (a) and (f), 0 h; (b) and (g), 1.75 h; (c) and (h), 2.75 h; (d) and (i), 3.75 h; (e) and (j), 2.75 h, with actinomycin added at 1.75 h. The cross-hatching indicates the shape of the theoretical curves fitted to the data, with the width of the cross-hatching representing one standard deviation from the curve of best fit.*

TABLE 1. *Deduced content of vRNA and cRNA in the nucleus and cytoplasm of an infected cell*[a]

Expt	Time of collection (h)	Time of actinomycin addition (h)	Time of cycloheximide addition (h)	Deduced cRNA/vRNA ratio in:		Deduced avg no. of viral genome equivalents in:					
						Nucleus		Cytoplasm		Whole cell	
				Nucleus	Cytoplasm	cRNA	vRNA	cRNA	vRNA	cRNA	vRNA
1	0			0.07 ± 0.02	0.07 ± 0.01	1 ± 1	6 ± 2	3 ± 1	42 ± 8	4 ± 1	48 ± 8
	1.75			1.33 ± 0.19	4.60 ± 0.84	219 ± 51	164 ± 69	490 ± 150	110 ± 58	709 ± 158	274 ± 90
	2.75			0.52 ± 0.06	2.85 ± 0.36	340 ± 139	644 ± 163	4408 ± 1496	1547 ± 825	4748 ± 1502	2191 ± 841
	3.75			0.26 ± 0.04	0.20 ± 0.03	127 ± 50	490 ± 105	1625 ± 690	8120 ± 1833	1752 ± 692	8610 ± 1836
	2.75	1.75		0.54 ± 0.09	0.39 ± 0.05	297 ± 141	552 ± 152	591 ± 309	1508 ± 483	888 ± 340	2060 ± 506
2	0			0.05 ± 0.02	0.05 ± 0.01	0 ± 0	1 ± 1	2 ± 1	31 ± 7	2 ± 1	32 ± 7
	2.5			0.44 ± 0.05	2.80 ± 0.39	93 ± 38	212 ± 79	1322 ± 543	472 ± 256	1415 ± 544	684 ± 268
	2.5		0	2.21 ± 0.24	1.96 ± 0.17	60 ± 14	27 ± 10	260 ± 53	133 ± 40	320 ± 54	160 ± 41
	2.5	0	0	0.72 ± 0.10	0.16 ± 0.04	27 ± 11	38 ± 9	7 ± 5	45 ± 16	34 ± 12	83 ± 18
	2.5	1	0	2.10 ± 0.28	0.37 ± 0.04	76 ± 22	36 ± 17	70 ± 26	190 ± 42	146 ± 34	226 ± 45
3	0			0.05 ± 0.01	0.07 ± 0.01	1 ± 1	15 ± 4	2 ± 1	35 ± 8	3 ± 1	50 ± 9
	2.5			0.53 ± 0.06	2.20 ± 0.26	140 ± 80	267 ± 82	3634 ± 1348	1652 ± 906	3774 ± 1350	1919 ± 82
	2.5		0	3.80 ± 0.57	2.65 ± 0.33	75 ± 16	20 ± 8	358 ± 82	135 ± 51	433 ± 84	155 ± 52
	2.5	0	0	2.50 ± 0.43	0.22 ± 0.04	40 ± 11	16 ± 8	17 ± 17	76 ± 8	57 ± 20	92 ± 11

[a] Cycloheximide and actinomycin D were used at 100 and 2 μg/ml, respectively. The method for deducing the ratio of cRNA/vRNA and the absolute average values of cRNA and vRNA sequences present per nucleus or cytoplasm is described in the text.

crease in cRNA after 2.75 h does not always occur; in other experiments the amount of cRNA remains essentially constant beyond 2.75 h. Other workers have noted the stability of viral cRNA (34).

Secondly, consider the distribution of viral sequences between nucleus and cytoplasm. The cRNA first detected at 1.75 h is present in both nucleus and cytoplasm, with approximately 30% being in the nucleus. At later times the absolute amounts in the nucleus do not increase significantly. In contrast, large increases in cRNA are detected in the cytoplasm, so that by 2.75 and 3.75 h about 95% of the total cRNA is in the cytoplasm. The increase in vRNA observed at 1.75 h occurs both in the nucleus and the cytoplasm. At later times, the amount of vRNA in the nucleus increases, but by no more than about fourfold, whereas an 800-fold increase is observed in the cytoplasm. Thus, at 3.75 h the distribution of vRNA is like that of cRNA in that about 95% of the vRNA sequences are found in the cytoplasm.

Effect of actinomycin on the synthesis of vRNA and cRNA. As part of the experiment described above, an additional infected-cell culture was treated with actinomycin at 1.75 h and harvested at 2.75 h. The kinetics of annealing obtained with RNA extracted from the nuclei and cytoplasm of these cells are also shown in Fig. 2, and the deduced amounts of vRNA and cRNA are included in experiment 1 of Table 1. Relative to the untreated controls, actinomycin added at 1.75 h blocked an otherwise ninefold increase in the yield of cRNA sequences in the cytoplasm. In contrast, actinomycin did not significantly affect the 14-fold increase of vRNA sequences in the cytoplasm. The amount of nuclear cRNA does not vary significantly in the period 1.75 to 2.75 h, and it is therefore not surprising that actinomycin added at 1.75 h has no effect on the amount detected in the nucleus at 2.75 h.

Primary transcription of the viral genome and the effect of actinomycin. Primary transcription is operationally defined as the transcription which occurs in the presence of CM added at zero time (3). Under these conditions, vRNA synthesis is suppressed (30, 33) and transcription is assumed to be restricted to copying of the inoculum vRNA by the inoculum transcriptase. To characterize the transcription that occurs under such conditions, we examined infected-cell cultures incubated for 2.5 h in the presence of CM at 100 μg/ml. As controls, two infected cultures, not treated with CM, were harvested at 0 and 2.5 h. The kinetics of annealing of the [^{125}I]vRNA and [^{32}P]CDNA to RNA extracted from the nuclei and cytoplasm of these cells is shown in Fig. 3. The amounts of vRNA and cRNA deduced using these data are shown in Table 1, as experiment 2. The deductions from an additional experiment with the same protocol are presented as experiment 3, and will be discussed with those of experiment 2.

Our assay reveals that the operational definition of primary transcription based on the use of high concentrations of CM needs some qualification. Although the total amount of vRNA detected at 2.5 h is reduced as a consequence of the addition of 100 μg of CM per ml at 0 h, the reduction is not absolute. Consequently, the cRNA detected under these conditions may not be exclusively transcripts of inoculum vRNA, but may also include some transcripts from newly synthesized vRNA. Nevertheless, it can still be presumed that this cRNA is enriched for transcripts resulting from genome primary transcription. As a consequence of the CM treatment the total amount of cRNA detected per cell is reduced by 80 to 90%. In the nucleus the reduction in cRNA is only about 40%, whereas in the cytoplasm the reduction is about 85%. The fraction of cRNA sequences detected in the nucleus increases from about 5 to 18% as a consequence of CM treatment.

Studies reported in the previous section indicate that actinomycin added at 1.75 h inhibits further synthesis of cRNA. The bulk of this cRNA probably represents the products of amplified transcription. Therefore, we undertook to determine the effect of actinomycin on primary transcription by studying its effect on that residual transcription that occurs in the presence of CM. For this purpose infected-cell cultures were treated not only with CM but also with actinomycin, the latter being added either at 0 or 1 h. The cells were collected at 2.5 h and fractionated into nucleus and cytoplasm. The kinetics of annealing in the presence of the extracted RNAs are included in Fig. 3 and deductions from these data are included in experiment 2 of Table 1. The deductions from a similar experiment are summarized as experiment 3 of Table 1. It can be seen that the addition of actinomycin at 0 h inhibits the synthesis of cRNA that occurs in the presence of CM by 85 to 90%. Most of this inhibition is seen in the cytoplasm where the amount of cRNA detected is reduced by at least 95%. In contrast, in the nucleus about 50% of the transcripts still appear. As a consequence, in such cells the majority, about 75%, of all cRNA sequences are found in the nucleus. The reduction in cRNA by actinomycin

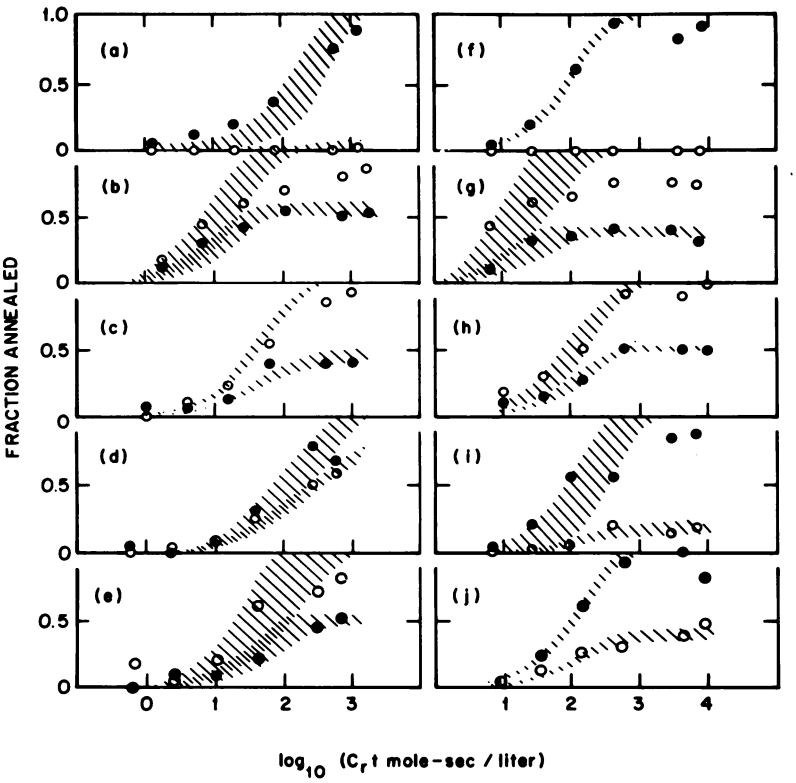

FIG. 3. *Annealing of labeled probes to RNA extracted from the nucleus and cytoplasm. The [^{32}P]cDNA (●) and [^{125}I]vRNA (○) were annealed in the presence of either nuclear RNA (a-e) or cytoplasmic RNA (f-j). After various values of C_rt, samples were removed and the fraction of the probes annealed was assayed by nuclease resistance. The infected cultures used were incubated at 37°C as indicated: (a) and (f), 0 h; (b) and (g), 2.5 h; (c) and (h), 2.5 h with CM added at 0 h; (d) and (i), 2.5 h with CM and actinomycin added at 0 h; (e) and (j), 2.5 h with CM added at 0 h and actinomycin added at 1 h. The cross-hatching indicates the shape of the theoretical curves fitted to the data. The limits represent one standard deviation from the curve of best fit.*

for the CM-treated cultures to which actinomycin is added at 1 h rather than at 0 h is 55% rather than 85 to 90%. In this case only those cRNA sequences detected in the cytoplasm are reduced. The amount of cRNA detected in the nucleus is not altered as a consequence of actinomycin added at 1 h.

DISCUSSION

The objectives of this study are twofold. First, we describe a technique based on the kinetics of annealing to specific labeled probes, which allows quantitation of influenza vRNA and cRNA sequences in infected cells. Secondly, we show how this method relative to previous methods reveals new information concerning the transcription and replication of the influenza virus genome.

The experimental assay as described uses [^{125}I]vRNA and [^{32}P]cDNA as probes. The specificity of these probes has been demonstrated. To determine the amount of cRNA and vRNA in a RNA fraction from infected cells, a measurement is made of the kinetics of annealing of the labeled probes in the presence of that RNA. The content of vRNA and cRNA in the infected-cell RNA is deduced by subjecting the experimental data to a theoretical analysis. Two assumptions are needed for this analysis. The first assumption is that the transcription of viral RNA is uniform. The vRNA present in the virion is actually composed of several unique segments (8, 19, 31), and it has been shown that throughout the course of infection the entirety of vRNA is transcribed (17). Nevertheless, it is not yet known whether or not each genome segment is transcribed with the same efficiency and also whether or not this pattern changes during the course of infection. As it stands, the analysis presented here is biased towards the detection of uniform transcripts, and if transcription is not uniform, the amount of cRNA would be underestimated (20).

The second assumption is that the rate constant for the annealng between unlabeled cRNA and vRNA in the RNA extracted from

infected cells is identical to that between these same sequences and the labeled probe. This is probably not the case experimentally, since the labeled probes are relatively smaller (modal sedimentation value 5S) than the viral species in infected cells and it is known that rate constants are dependent upon size (39). Since the probes are of comparable size, they should be at an equal disadvantage. A possible additional complication is that the labeled cDNA probe, being composed of DNA rather than RNA, might have a different rate constant. We are unaware of any data comparing rate constants for DNA-RNA versus RNA-RNA annealing. Straus and Bonner (37) have shown that the rate constant for DNA-DNA annealing is larger than that for DNA-RNA. It is therefore possible that the rate constant for DNA-RNA is larger than that for RNA-RNA annealing. Such a difference would cause overestimates of vRNA sequences due to the use of a DNA probe.

In summary, the possible consequences of one or more of our assumptions being invalid would be that the estimated amount of cRNA sequences could be underestimated relative to vRNA. The amount of vRNA might be either an under- or overestimate. It was noted in the Results that in certain cases the data do not fit well to the theoretical formulation. A possible reason for such deviation might be that in such situations the transcription of the viral genome is not uniform. Studies using labeled probes for individual genome segments should be able to test this hypothesis. Such systematic errors should not affect our qualitative conclusions made regarding the replication and transcription of the influenza viral genome.

Other workers using techniques different from those described here have undertaken to characterize the synthesis of virus-specific RNA in influenza virus-infected cells (3, 30, 33). Scholtissek and Rott (33) extracted total pulse-labeled RNA from infected cells and measured the ability of an excess of unlabeled vRNA or of an RNA fraction enriched for cRNA, to anneal to that labeled RNA and thereby protect it against a subsequent RNase digestion. A difficulty in their study was that pulse-labeled RNA from uninfected cells demonstrated some nuclease resistance both before and after annealing to unlabeled viral RNA, and, to compensate, a value proportional to this background was subtracted from their data for infected-cell RNA. In this way they deduced the time course of appearance of vRNA and cRNA in chicken embryo fibroblasts infected by fowl plague virus. The results are qualitatively similar to those presented here for the infection of canine cells by the WSN strain of influenza virus. That is, the synthesis of cRNA precedes that of vRNA, whereas at later times vRNA synthesis prodominates. In addition, using cell fractionation, we have been able to follow the relative distribution of viral sequences between nucleus and cytoplasm. An early increase in vRNA and cRNA sequences is detected in the nucleus, but as the infection proceeds,the majority of these sequences is detected in the cytoplasm. Thus, for cRNA sequences, the percentage in the nucleus decreases from 30% at 1.75 h to 5% at 2.75 h and later. In light of the results obtained concerning the cRNA synthesized in the presence of CM, it will be of interest to determine whether at times before 1.75 h an even larger fraction of the cRNA synthesized in the absence of CM is in the nucleus.

The effect of actinomycin on virus-specific RNA synthesis has been studied by Scholtissek and Rott (33) and by Pons (30). The former workers using the techniques described above found that actinomycin added at 2 h inhibited cRNA synthesis by 95% and vRNA synthesis by 70%. A similar preferential inhibition of cRNA synthesis was observed by Pons (30) using a different technique. The results presented here are in general agreement with these earlier studies but differ in that an even greater specificity for this drug is demonstrated. The increase in cRNA in the cytoplasm is blocked completely by actinomycin, whereas there is no detectable effect on the increase in vRNA in the cytoplasm. Our interpretation of these results is that there is an actinomycin-sensitive nuclear step required for the appearance of cRNA in the cytoplasm. As the product of this nuclear step could function in either the nucleus or the cytoplasm, the actual site of cRNA synthesis cannot be deduced from these results.

The first molecules of cRNA to appear in the infected cells must arise by what is called primary transcription, that is, transcription of the inoculum vRNA by the inoculum virion transcriptase. The procedures of Scholtissek and Rott (33) and of Pons (30) are not sufficiently sensitive to detect virus-specific synthesis at early times, before 2 h, and would not be expected to be capable of detecting primary transcripts. Consequently, Bean and Simpson (3) used a different approach to characterize such transcription by infecting cells with virus radioactively labeled in its RNA. They extracted the total RNA from the infected cells, denatured the RNA and subjected it to annealing conditions, and then determined what fraction of the labeled inoculum vRNA had been converted to a RNase-resistant form. The amount of this RNase resistance was used as a measure of the amount of transcription occurring in the in-

fected cells. By this technique they were able to detect transcription in the presence of CM (20 g/ml), but were unable to detect transcription in infected cells treated with both CM and actinomycin. In contrast to this latter result, we find that a significant fraction of the cRNA synthesized in the presence of CM is not suppressed by actinomycin and, furthermore, that the residual cRNA is primarily detected in the nucleus. It is probable that the inability of Bean and Simpson (3) to detect such residual transcription reflects a relative lack of sensitivity in their assay. This may have arisen from an inadequate combination of RNA concentration and annealing time and maybe also from an inadequate molar excess of newly synthesized cRNA relative to labeled inoculum vRNA. In their studies the maximum extent of annealing of labeled vRNA with RNA extracted from cells at 3 h after infection in the presence of CM was only 25%, rather than 100% which would be expected for vRNA completely transcribed into cRNA. In constrast, with our assay [^{125}I]vRNA was rendered 75 to 80% resistant to RNase as a consequence of annealing to RNA from CM-treated cells (Fig. 3).

Our results concerning the effect of actinomycin on the synthesis of cRNA occurring in the presence of CM provide some insights into the process of primary transcription. The addition of CM alone at zero time leads to an increase in the percentage of the cRNA detected in the nucleus, and when actinomycin as well as CM is added at zero time the cRNA that continues to be synthesized is found almost totally in the nucleus. The latter nuclear cRNA represents 50% of that detected in the presence of CM alone and 30% of that detected in the absence of both inhibitors. Thus, in the presence of both inhibitors, a significant fraction of the cRNA present in the nucleus during a productive infection persists. In contrast, almost no cRNA appears in the cytoplasm in the presence of both inhibitors. Our interpretation of these observations is that the cRNA that continues to appear in the nucleus in the presence of these two inhibitors results from transcription of the inoculum vRNA by the inoculum virion transcriptase, that is, primary transcription. This interpretation is consistent with published studies which indicate that the virion-associated transcriptase as studied in vitro is not inhibited by actinomycin (7, 27, 36). If the primary transcription of cRNA occurs in the nucleus of actinomycin-treated cells, it remains to be explained why cRNA no longer appears in the cytoplasm. One possibility arises from recent in vitro studies of the virion-associated transcriptase. It has been found that in vitro transcripts contain little or no polyadenylic acid [poly(A)] and are normally small relative to the vRNA template (5, 16, 22a). However if certain specific primers oligonucleotides are added during such transcription, the products obtained contain poly(A) and are much larger, in fact comparable in size to cRNA isolated from infected cells (22a, 28a). It can therefore be suggested that the transcription which occurs in infected cells treated with actinomycin is actually nonfunctional mRNA, that is poly(A)-deficient and small. This could arise because actinomycin reduces the availability of essential primers and the transcripts made under such conditions fail to migrate to the cytoplasm. This interpretation can be tested.

It is to be emphasized that our results do not give any direct evidence concerning the site of synthesis of cRNA that arises from amplified transcription, as opposed to primary transcription. Most of the cRNA transcribed in the absence of CM is detected in the cytoplasm. The appearance in the cytoplasm of the transcripts, as with primary transcripts, is inhibited by actinomycin but this does not establish the site of synthesis. With respect to the site of synthesis, it is significant that this cRNA has been found to contain internal N^6-methyladenosine residues (23). To date, such modified bases have been detected only in mRNA's synthesized in the nucleus but not in those viral mRNA's that are synthesized in the cytoplasm (1, 9, 12, 13, 25, 35). Therefore, it can be suggested that influenza viral cRNA, rather than being an exception, may actually be synthesized in the nucleus.

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