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Use of specific single stranded DNA probes cloned in M13 to study the RNA synthesis of four temperature-sensitive mutants of HK/68 influenza virus

Françoise Thierry* and Olivier Danos+

*Unité d'Ecologie Virale, and +Unité des Virus Oncogènes, Institut Pasteur, 25, rue du Dr. Roux, 75724 Paris, France

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ABSTRACT

Specific single stranded DNA probes have been obtained for both influenza virion RNA (vRNA) and complementary RNA (cRNA) by cloning a hemagglutinin gene fragment in the single stranded DNA phage M13. These probes were used for hybridization with the total labeled RNA from cytoplasmic extracts of infected cells. MDCK cells were infected with temperature-sensitive mutants of Influenza HK/68 and the production of the virus specific RNA species was analysed at both permissive and restrictive temperatures. Results show that two NP mutants which undergo intracistronic complementation exhibit two different phenotypes at the non permissive temperature: ts2C is poly A cRNA and vRNA negative whereas ts463 is RNA positive. Two mutants of P genes were also analysed and we discuss the relationship existing between the synthesis of the three RNA species especially between poly A and non poly A cRNA.

INTRODUCTION

A major problem in the study of Influenza virus replication and transcription is that virus specific RNA represents a very small fraction of the total RNA in infected cells. The inhibitors of cellular transcription such as Actinomycin D inhibit also the virus multiplication (1, 2, 3) and therefore cannot be used to suppress the cellular background. The problem is further complicated by the fact that both vRNA and cRNA are present together in the infected cells. Previous studies (4, 5) to characterize the influenza RNA synthesis have been undertaken with two specific radiolabeled probes to the virus specific vRNA and cRNA. Measurement of kinetics of annealing of these probes to the RNA of infected cells gave an estimate of the number of copies of each virus specific RNA in the cells. In this paper, we describe a new approach to study influenza RNA synthesis which is based on the use of specific non radiolabeled single stranded DNA probes obtained from the hemagglutinin gene fragment. To obtain these probes, we cloned in the replicative form of the filamentous coliphage M13 a 2000bp

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Bam HI fragment previously purified from the plasmid pXHA/14 carrying a double stranded cDNA copy of the hemagglutinin gene (6). The cloned fragment contains 800 b of the hemagglutinin insert. This cloning method permitted a massive and homogeneous production of single stranded DNA probes complementary to the minus and plus viral RNA. A large number of studies with temperature-sensitive mutants of Influenza virus (7, 8, 9, 10, 11) have been undertaken to elucidate which polypeptides are involved in RNA synthesis. With exception of the study of one FpV ts mutant (12), none of these experiments examined independently the synthesis of the three viral RNAs. We used our probes immobilized on nitrocellulose filters and in a hundred fold molar excess to measure ^{3}H-adenosine labeled poly A cRNA, non poly A cRNA and vRNA in influenza infected cells. This method allowed a rapid and specific detection of each RNA species in cells infected with temperature-sensitive mutants at both permissive and non permissive temperatures. We have been able to determine the complete phenotype at the non permissive temperature for two ts mutants of the P1 and P2 genes and two mutants of the NP gene which have been shown to undergo intracistronic complementation (13, 14).

MATERIALS AND METHODS

Cloning of the pXHA/14 fragment in M13 mWJ43. The pXHA/14 recombinant pBR322 plasmid containing a full length copy of the hemagglutinin gene of H3 subtype has been kindly provided by Dr. W. Fiers. The restriction map and the sequence of this plasmid have been previously described (6, 15). A 2000 bp Bam HI restriction fragment containing 800 bp of the HA insert was prepared. After complete digestion of 15 µg of pXHA/14 with Bam HI, the 2000 bp fragment was purified by electrophoresis on a 4% acrylamide gel and recovered by electroelution. This fragment was added to the Bam HI linearized replicative form of M13 mWJ43 (16) in a molar ratio of about 10 to 1. Ligation with 0,8 unit of T4 DNA ligase was performed at 4°C overnight. The ligated DNA was used to transform E. coli JM101 [Δ (lac pro) sup E, thi F' tra D26 pro AB lac I^{q} ZΔM15] by the calcium chloride technique (17). Recombinant phages were isolated under IPTG induction with X Gal as substrate (18) and further grown for characterization.

Characterization of the DNA orientation. Preparation of M13 virions from the supernatant of a JM101 culture infected with a colorless plaque, and purification of single stranded DNA from these virions have been previously described (19, 20). Single stranded DNA with the 2000 b insert were identified

by running an aliquot on agarose gel. Orientation of the cloned fragment in M13 recombinants was determined by an hybridization test as described elsewhere (20). Representative recombinant clones of each orientation were furthere characterized by hybridization of their DNA with vRNA extracted from purified virus. They were also hybridized to labeled vRNA (extracted from ^{3}H labeled virus) and the extent of hybridization was deduced after digestion with RNase A (10 µg) in 0,3 M NaCl.

Labeling of influenza RNA in vivo. MDCK confluent cells grown in 145 mm Petri dishes were infected by the HK/68 (H3N2) influenza virus or temperature-sensitive mutants of this strain at a multiplicity of infection of 5-10 pfu/cell. After an adsorption period of 30 mn at room temperature, the cells were incubated at either 32°C (permissive temperature) or 39°C (non permissive temperature) with 10 ml prewarmed medium (Leibowitz). Infected cells were labeled with ^{3}H adenosine (Amersham) at 10 µCi/ml. At the end of the incubation period, cells were washed 3 times with cold PBS, pelleted and lysed with 0,5% NP40 followed by 10 strokes with a Dounce homogeneizer. The nuclei were pelleted and the RNA of the supernatant extracted 3 times with a phenol-chloroform mixture equilibrated at pH 9. The RNA was precipitated with ethanol. RNA concentrations were determined by the absorption at 260 nm. Radioactivity was measured after TCA precipitation of an aliquot and filtration on GFC filters in a toluene based scintillation fluid.

Fractionation of cytoplasmic RNA on oligo-dT cellulose. Cytoplasmic labeled RNA were fractionated into non poly A and poly A containing RNA on an oligo-dT cellulose column as previously described (21).

DNA-RNA hybridizations with the specific DNA probes. Hybridizations were performed as described (22, 23) with some modifications (no alkaline denaturation of the single stranded probes was required). Nitrocellulose filters (Schleicher & Schüll, BA 85 0.45µ) were rinsed once with H_2O and once with 6 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate) prior to filtration of DNA diluted in 6 x SSC. 5 to 10 µg of recombinant phages DNA in both viral and complementary orientations were immobilized on each filter. Filters, cut into quarters, were baked for 2 hrs at 80°C. Each quarter was placed in an Eppendorf tube containing ^{3}H labeled RNA of the cytoplasmic extracts of infected cells in 0,2 ml of (10 mM Tris pH 7,4; 0.5 M NaCl) and 0,3 ml of 1 x SSC. Hybridizations were carried out overnight at 68°C. Filters were washed with 2 x SSC, incubated for 30 mn with 10 µg of RNase A in 1 ml of 2 x SSC, rinsed, dried and their radioactivity determined in scintillation fluid.

Temperature shift experiments. MDCK cells in 60 mm Petri dished were infected with 5 to 10 pfu/cell of HK ts mutants. After an adsorption period of 30 mn at room temperature, they were rinsed 3 times, overlaid with prewarmed Leibowitz medium containing 0.5 µg of trypsin and incubated at 39°C. At different times infected plates were transferred from 39°C to 32°C (shift down) and further incubated for a total of 10 hours post infection. Virus yield was measured by plaque formation on MDCK cells at the permissive temperature.

RESULTS

Construction and characterization of the M13 clones. Cloning of the Bam HI fragment from pXHA/14 in M13 mWJ43, isolation of recombinant phages and characterization of their relative orientations were carried out as described in Materials and Methods. Five recombinants were studied, two of them were in one orientation and three in the opposite orientation, we chose two recombinants representative of each orientation. About 100 µg of single stranded DNA from these recombinant phages were prepared from the supernatant of a 50 ml culture. 0.1 µg of each of the DNA preparations was hybridized to increasing concentrations of viral RNA extracted from the purified virus in the conditions described for DNA-DNA hybridization in ref. (20). On a 1% agarose gel, the migration of the HA_1-4 recombinant clone is slowed down by hybridization with the vRNA while HA_1-2 is not affected (fig. 1). Thus, HA_1-4 will be refered to as a cDNA probe (complementary to vRNA) to study labeled influenza vRNA synthesis and HA_1-2 as a vDNA probe (viral orientation) to study labeled influenza cRNA synthesis. We also determined in this study that the complete hybridization of 0.1 µg of the cDNA probe requires more than 1 µg of vRNA (fig. 1 lane 5). This is in agreement with the fact that this probe only hybridizes with the HA gene which represents about 14% of the total genome (∿ 1768 b/∿ 15,000 b). In addition, we studied the hybridization of the DNA probes to a preparation of pure labeled vRNA (vRNA extracted from purified virus labeled with ^3H adenosine (specific radioactivity 70,000 cpm/ µg of RNA) followed by a RNase A digestion in 0.3 M NaCl. The maximum of protection of of the labeled vRNA at a saturating concentration of cDNA [i.e. > 0.01 µg for 0.1 µg of labeled vRNA (7000 cpm)] is 6% of its total radioactivity. This percentage in is agreement with the fraction of the influenza genome cloned in M13 (800 b/∿ 15,000 b).

Specificity of the DNA-RNA hybridization method. Prior to investigating the synthesis of influenza RNA in infected cells, we studied the specificity

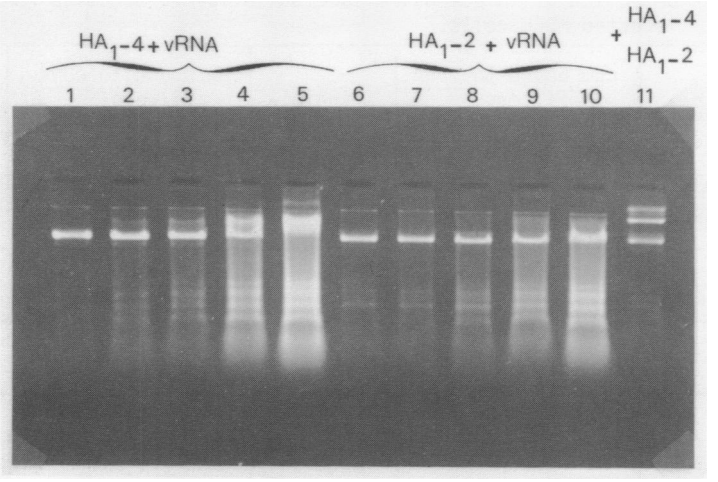

Figure 1 Electrophoretic analysis on a 1 % agarose gel of the hybrids obtained by vRNA-DNA hybridization (lanes 1 to 10) and DNA-DNA hybridization (lane 11). - The recombinant phages HA_1-4 and HA_1-2 DNAs were hybridized with increasing concentrations of vRNA for 1 hour at 68° C, then directly analysed on the gel. Migration was for 1 hr at 150 volts.
Lanes 1 to 5 : 0.1 µg of the HA_1-4 DNA is hybridized with 0.01 ; 0.05 ; 0.1 ; 0.5 ; 1 µg of pure vRNA.
Lanes 6 to 10 : same protocol with HA_1-2 DNA.
Lane 11 : hybridization of 0.05 µg of both HA_1-4 and HA_1-2 DNAs.

of the DNA-RNA hybridization method described in Materials and Methods. This method allows the analysis of a mixture of cRNA and vRNA by binding each of these species to specific filters: both cDNA and vDNA filters are added to each labeled cytoplasmic RNA sample. However, this determination will be quantitative only if the RNA-RNA hybridization in the aqueous phase is minimized. This is achieved under two hybridization conditions: 1) the quantity of DNA bound to the nitrocellulose filter is much higher than the concentration of virus specific RNA in the sample; 2) the RNA in the aqueous phase is diluted and fragmented. Hence, in most of our assays, the DNA bound to filters represented a hundred fold molar excess over the homologous virus specific RNA. The results summarized in Table I demonstrate the effect of adding pure ^3H labeled vRNA on the percentage of cRNA hybridizing to the DNA filters. When the assays were performed with 10 µg or less of total cytoplasmic RNA, the virus specific RNA's hybridized quantitatively and specifically to DNA filters. Using these concentrations no RNA-RNA hybridization was detected in the aqueous phase. For 10 to 20 µg (20,000 to 100,000 cpm) of cytoplasmic RNA, the

Table I - Detection of ^{3}H adenosine labeled influenza specific RNA's in infected MDCK cells.

Concentration (µg/ml) of cytoplasmic RNA*	cpm bound to vDNA filters		% inhibition of cRNA binding	cpm bound to cDNA filters		% inhibition of vRNA binding
	no vRNA added	+^{3}HvRNA$^\Delta$		no vRNA added	+^{3}HvRNA$^\Delta$	
0	-	20	-	-	250 ▼	0
8	260	300	0	35	290	0
16	490	340	30	29	120	54
32	970	720	26	40	100	62

* The cytoplasmic RNA was labeled from 2 to 4 hrs post infection at 32°C. In this early pulse labeled sample vRNA was not detectable.

Δ ^{3}HvRNA was extracted from purified virus labeled with ^{3}H adenosine, 5000 cpm were added per sample (0.1 µg of RNA).

▼ The fraction of vRNA homologous to the cDNA probe is 6% (∿ 800 b/∿ 15,000 b).

amount of specific RNA hybridized on filters was from 500 to 3,000 cpm. The variability observed depended on the experimental conditions such as time and temperature of incubation, labeling, confluence of the cells and virus inoculum. However, the lowest limit of detection is about 50 cpm/filter which represents the background of binding of cytoplasmic RNA from non infected cells.

Characterization of the production of the HK influenza specific RNA species in MDCK infected cells under permissive conditions. To label the cytoplasmic RNA, 100 µCi of ^{3}H adenosine were added per plate at 2 hours after infection to several infected MDCK plates which were then incubated for different periods. For the first two hours after infection, the virus specific RNA synthesis is not detectable at 32°C and is very low at 39°C. Furthermore, after this time, the shut off of cellular RNA synthesis is efficient as we have determined in independent experiments in which we have analysed poly A RNA from infected cells on polyacrylamide gels (results not shown). At different times after infection, labeled plates were removed and their RNA extracted

from the cytoplasmic fraction. The cytoplasmic RNA's were fractionated into two populations of poly A and non poly A RNA using an oligo-dT cellulose column as described in Materials and Methods. Quantitative accumulation of specific vRNA and cRNA were analysed in each cytoplasmic RNA sample by hybridization with cDNA and vDNA filters as described. The non poly A RNA fraction contains both virus specific cRNA and vRNA as shown on figures 2 and 3. The first RNA species detectable is the non poly A cRNA which accumulates but decreases which indicates the occurence of a specific degradation; this phenomenon has been previously observed by Taylor et al (4). Analyses using one hour pulse labeling at different times after infection show, indeed, that the maximum rate of synthesis is between 4 and 5 hours pi and that after this time the non poly A cRNA synthesis is stopped (results not shown). The maximum accumulation of vRNA occurs very late, increasing sharply afters 5 hours pi similarly to the poly A cRNA (fig. 2). The comparative studies of the RNA synthesis in different ts mutants at the permissive temperature indicate that the accumulation patterns of vRNA and poly A cRNA are slightly different from one mutant to another. Mutants ts 454 (fig. 3) and ts 463 (not shown) exhibit a higher rate of production than ts 315 (fig. 2) and ts 2C (not shown). In addition vRNA and poly A cRNA syntheses are closely coupled in the second pair of mutants. On the other hand, the synthesis of non poly A cRNA is similar for the different strains and behaves independently of the two other species of RNA.

Analysis of the RNA sysnthesis of 4 Influenza HK temperature-sensitive mutants at non permissive temperature. We studied the RNA synthesis at non permissive temperature of 4 temperature-sensitive mutants of the influenza HK 68 strain that we have previously analysed genetically (13, 14, 25). Their mutations are localized on RNA fragments 2 (ts 315), 3 (ts 454) and 6 (ts 2C and ts 463). We analysed the rate of accumulation of the 3 RNA species in the cytoplasm of infected cells at the non permissive temperature (39°C) and compared it to that observed at the permissive temperature (see figs. 2 & 3). The results are summarized in Table II. Mutant ts 315 exhibits a low level of synthesis of the 3 RNA as shown in figure 2. It is of interest to compare these results with those of ts 454 and try to define more precisely their defects. For ts 454, the accumulation of non poly A cRNA is normal at any time after infection while the synthesis of vRNA and poly A cRNA show a marked decrease. For both mutants, as shown in figures 2 and 3, the RNA synthesis is normal until 4 hours pi, however, the sensitivity of our assay does not allow an unambiguous interpretation of this point. In experiments involving tempera-

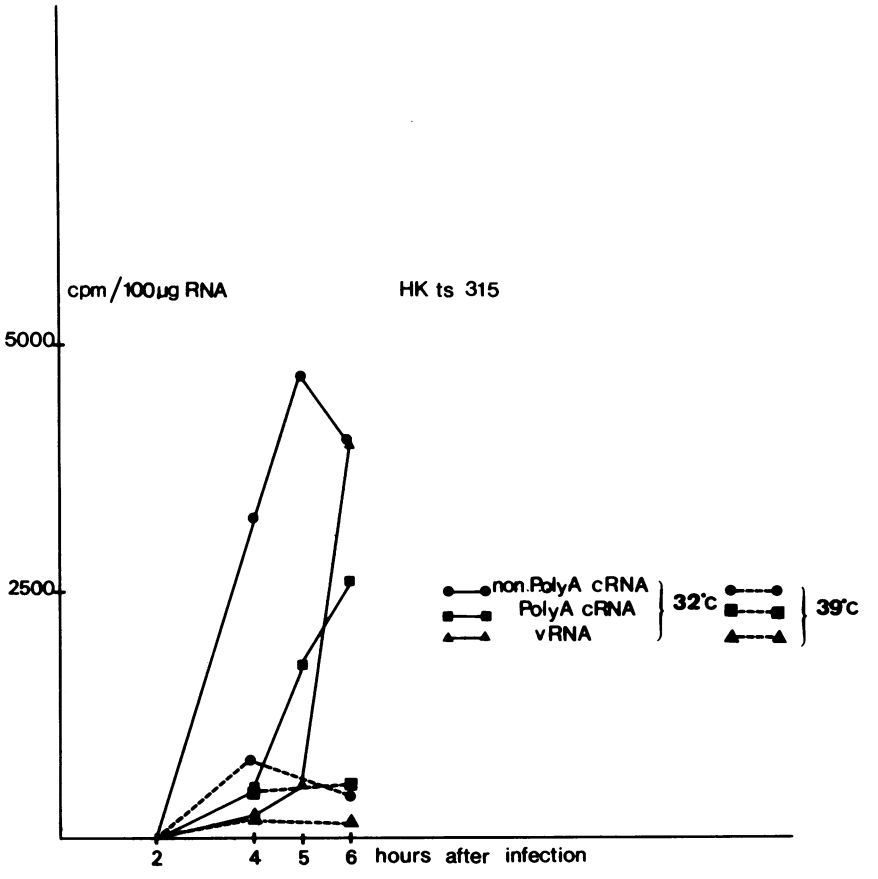

Figure 2 Analysis of the influenza RNA's from the cytoplasm of MDCK cells infected with the HK mutant ts 315 at 32°C and 39°C. The labeled cytoplasmic RNA fractionated into poly A RNA and non poly A RNA on an oligo-dT cellulose column were analysed by hybridization on nitrocellulose filters containing cDNA and vDNA probes. The cpm bound on filters at the end of the hybridization time are normalized to 100 µg of total cytoplasmic RNA.

ture shift (see fig. 4), these two mutants behave like "late mutants". Temperature shift up experiments confirm this result (data not shown). The temperature-sensitive steps in the production of infectious virus occur at about 4-5 hours post infection. Therefore, for both mutants, the early events including the primary transcription, are not affected at the non-permissive temperature. From these results, it is possible to delineate the defects of ts 315 and ts 454 in the virus replication: mutant ts 315 exhibits a defect

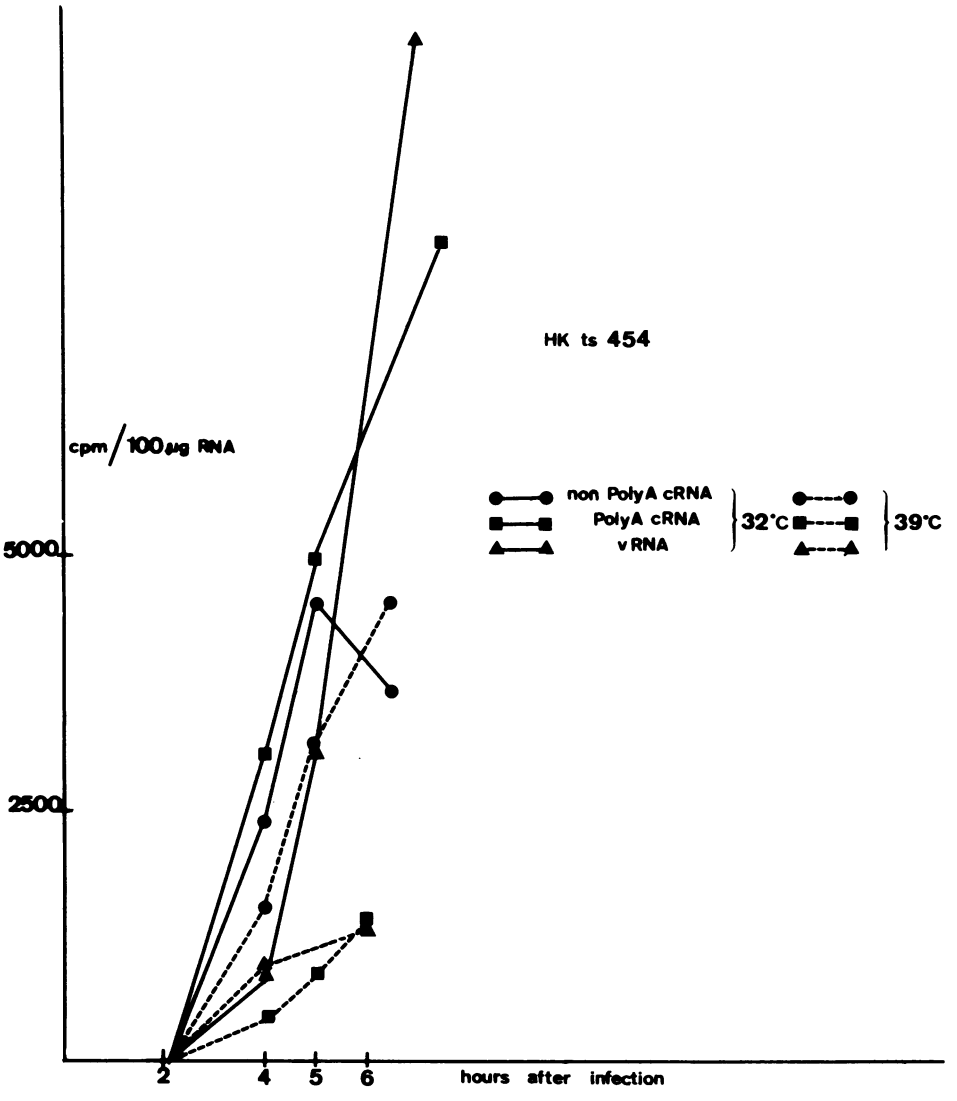

Figure 3 Analysis of the influenza RNA's from the cytoplasm of MDCK cells infected with the HK mutant ts 454 at 32° C and 39°C.

in the production of non poly A cRNA which entails defects in the late synthesis of vRNA and subsequent amplification of poly A cRNA. Mutant ts 454 appears to be a true mutant of replication which is not able to synthesize vRNA after a normal production of non poly A cRNA template. In temperature shift experi-

Table 2 - RNA phenotypes of HK 68 ts mutants at 39° C : RNA synthesis of the 3 species was studied as described in the text.

	RNA fragment	Polypeptide	Poly(A)cRNA	Non poly(A)cRNA	vRNA
ts 315	2	P_1	Reduced[b]	Reduced	Reduced
ts 454	3	P_2	Negative[c]	Normal[a]	Negative
ts 2C	6	NP	Reduced	Normal	Negative
ts 463	6	NP	Normal	Normal	Normal

(a) 50 to 100 %
(b) 20 to 50 % } of the synthesis at permissive temperature.
(c) 0 to 20 %

ments, ts 2C and ts 463 exhibit a pattern similar to that shown in figure 4 for ts 315 and ts 454. However, the analysis of their RNA synthesis at the non permissive temperature gives different phenotypes (Table II). ts 2C seems to behave as a true mutant of virus replication like ts 454, while ts 463 does not show any defect in RNA synthesis and can therefore be considered as a mutant of maturation. They are both mutants of the nucleoprotein gene. This shows clearly that this polypeptide is multifunctional (replication + maturation), as previous results on intracistronic complementation already suggested (13, 14).

DISCUSSION

The objectives of this study are twofold. First, we describe a technique based on the specific annealing to single stranded DNA probes which allows the analysis of the three influenza RNA species in the infected cells. Secondly, we show how this method can be used for the rapid analysis of the RNA phenotypes of temperature-sensitive mutants. The assay used non labeled single stranded DNA's of both orientations as probes. The specificity of these probes has been demonstrated. To determine the relative amount of poly A cRNA, non poly A cRNA and vRNA in infected cells, a measurement of the annealing of

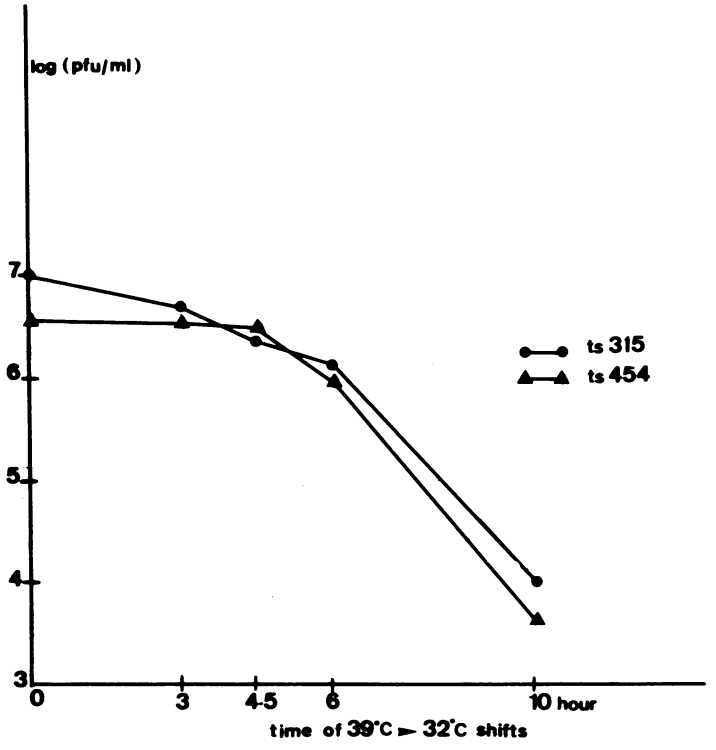

Figure 4 Temperature shift down: virus yield of MDCK cells infected with ts 315 and ts 454 at 39°C and kept for various times at this temperature before being transferred to 32°C.

labeled cytoplasmic RNA to nitrocellulose filters containing single-stranded DNA probes was made. This analysis requires large amounts of pure cDNA and vDNA, two conditions which are achieved by cloning in the single stranded DNA phage M13. In this assay, analysis of the results are both direct and simple. They do not require any mathematical extrapolation as did the previous studies using specific probes (4, 5). Thus, it is well adapted to a large scale comparative analysis of several mutants both at permissive and restrictive temperatures at different periods after infection. Since the influenza specific sequence of the DNA probes contains part of the hemagglutinin gene, one assumption is required for this analysis: the transcription and replication of this gene is representative of the complete genome. The annealing analysis of specific probes of each gene have demonstrated a quantitative and temporal control of the messenger RNA's (poly A cRNA) synthesis (5), HA is a late gene which is

transcribed in a roughly tenfold lower copy number than the M or NS genes. However, there is no reason to postulate that the mRNA synthesis for the other genes is affected differently by the mutations which have been studied. Non poly A cRNA synthesis is not under the same control, since the segments are synthesized in equimolar amounts. For the vRNA synthesis, the situation is unclear, it has been postulated that the non equimolar synthesis of the vRNA segments in infected cells controls the synthesis of mRNA (26). Little is known about this control and the analysis of the vRNA synthesis with the assay described in this paper, using in parallel specific probes constructed from other genes, should clarify this question.

Our results, in which the relative accumulation of the 3 RNA species was determined at different times after infection in the permissive or non permissive temperatures are in agreement with previous studies on the WSN (H_0N_1) strain (4) and avian FpV strain (5). Although our assay does not allow a quantitative measurement of the RNA accumulations (in genome equivalents as did the previous studies cited), the kinetics of relative accumulation of RNA species are very similar . Analysis of the mutants ts 315 and ts 454 shows that, indeed, P1 and P2 are involved in RNA synthesis as previously demonstrated (27, 28, 29). Unfortunately, we could not extend our study to P3 because we did not possess any conveient HK mutant of this gene. P1 has been demonstrated to be involved in cRNA synthesis (27, 28) and the present study specifies this point showing that it regulates non poly A cRNA rather than poly A cRNA synthesis. P2 is involved in vRNA synthesis as previously described for the (H_0N_1) WSN strain (27, 28). The complete phenotype of the ts 454 (P2) mutant demonstrates that the synthesis of the non poly A cRNA does not depend on the increase in the synthesis of vRNA, while it is the case for the poly A cRNA. Two observations can be made from the results concerning the synthesis of the 2 classes of cRNA. First, they are synthesized independently. Secondly, poly A cRNA accumulation is dependent on vRNA synthesis late in infection. Thus the vRNA templates for the two cRNA species are different. The nucleoprotein has been demonstrated to be involved in the vRNA synthesis (27, 30). Our results show that this is not the only function of this polypeptide. While ts 2C behaves as a replication mutant, ts 463 does not exhibit any characteristic defect in the RNA synthesis (Table II). This is in agreement with the observation that intracistronic complementation can occur among NP mutants (13, 14). The NP polypeptide is the backbone of the viral structure and is certainly involved in several essential functions in the virus multiplication. This study emphasizes this last point. It will be of interest to extend the analysis of

RNA synthesis to other ts mutants of the same gene. This would permit to define precisely the multiple functions of the viral genes.

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