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In vitro synthesis of full-length influenza virus complementary RNA

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Influenza virus-specific RNA has been synthesized *in vitro*, using cytoplasmic or microsomal fractions of influenza virus-infected MDCK cells. The RNA polymerase activity was stimulated 5 – 30 times by priming with ApG. About 20 – 30% of the product was polyadenylated. Most of the *in vitro* product was of positive polarity, as shown by hybridization to strand specific probes and by T1 fingerprinting of the poly(A)$^+$ and poly(A)$^-$ RNA segments encoding haemagglutinin and nucleoprotein. The size of poly(A)$^-$ RNA segments, determined on sequencing gels, was indistinguishable from that of virion RNA, whereas poly(A)$^+$ RNA segments contain poly(A) tails ~50 nucleotides long. The size of *in vitro* synthesized RNA segments was also determined by gel electrophoresis of S1-treated double-stranded RNAs, obtained by hybridization of poly(A)$^+$ or poly(A)$^-$ RNA fractions with excess of unlabelled virion RNA. The results of these experiments indicate that poly(A)$^-$ RNA contains full-length complementary RNA. This conclusion is further substantiated by the presence of additional oligonucleotides in the T1 fingerprints of *in vitro* synthesized poly(A)$^-$ haemagglutinin or nucleoprotein RNA, selected by hybridization to cloned DNA probes corresponding to the 3′ termini of the genes.

Key words: RNA replication/T1 fingerprinting/molecular cloning/hybridization

Introduction

The genome of type A influenza virus consists of eight single-stranded RNA segments of negative polarity (Palese and Kingsbury, 1983). Three types of virus-specific RNA are synthesized in influenza virus-infected cells: (i) mRNA carries cell-derived cap-containing primers and poly(A) (Krug *et al.*, 1979; Caton and Robertson, 1980; Robertson *et al.*, 1981; Beaton and Krug, 1981); (ii) complementary RNA (cRNA) is a complete copy of parental vRNA, contains ATP at its 5′ end and is not polyadenylated (Hay *et al.*, 1982). Because of its structure, cRNA is supposed to serve as template for the synthesis of progeny virion RNA; (iii) virion RNA (vRNA) synthesis follows that of cRNA and takes place at a different rate for each RNA segment (Smith and Hay, 1982).

Purified influenza virions include an RNA polymerase activity (Bishop *et al.*, 1971; Chow and Simpson, 1971; Skehel, 1971), which can be stimulated by priming with ApG (McGeoch and Kitron, 1975; Plotch and Krug, 1977) or with some cap-containing mRNAs (Bouloy *et al.*, 1978). An RNA polymerase activity has also been detected both in the nuclear and in the microsomal fractions of infected cells (Ho and Walters, 1966; Mahy and Bromley, 1970; Ruck *et al.*, 1969; Scholtissek and Rott, 1969; Skehel and Burke, 1969; Hastie and Mahy, 1973). The product of this activity induced in infected cells is mainly RNA of positive polarity but has not been characterized in detail. Very recently, however, Beaton and Krug (1984) described an *in vitro* system derived from the nuclei of influenza virus infected cells, able to synthesize both mRNA and cRNA.

Here we describe preparations of viral specific RNA polymerase derived from cytoplasmic or microsomal fractions of MDCK infected cells, able to synthesize full-length mRNA and cRNA *in vitro*.

Results

RNA polymerase activity

In our efforts to search for *in vitro* replicative synthesis of influenza virus RNA, we have studied the RNA polymerase activity induced in MDCK cells after infection with A/Victoria/3/75 virus. Induced enzyme could be detected first 3 h post-infection (h.p.i.), both in the nuclear and in the cytoplasmic fractions of the infected cells and reached its maximum activity at 5 – 7 h.p.i. In both fractions, the RNA polymerase activity increased 5 – 30 times when ApG was used as primer, although this stimulation factor was smaller with longer incubation times, in agreement with previous results (Hay and Skehel, 1979). The incorporation showed linear kinetics for 20 min, in the presence of ApG, with similar incorporation rates at 30, 37 or 39°C (data not shown). About 20% of total activity was present in the nuclear fraction, both when the cells were opened with Nonidet P-40 (NP-40)-containing HB buffer or by Dounce homogenization in the absence of detergent. The enzyme present in the cytoplasmic fraction was partially purified either by gel filtration or differential centrifugation. The specific enzymatic activity of these partially purified preparations reached values up to 7 pmol UMP incorporated per μg of protein, similar to those estimated for purified virions (Plotch and Krug, 1977; Hay and Skehel, 1979).

Characterization of the RNA product

Oligo(dT)-cellulose chromatography. In vitro synthesized RNA was fractionated through oligo(dT)-cellulose columns. About 20 – 30% of the total RNA was retained by the column, both when the excluded volume of Sephadex G200 or the microsomal pellet was used as a source of enzyme.

Polarity of the in vitro product. The polarity of the *in vitro* product was assayed by hybridization to the strand-specific probes described in Materials and methods. When these probes were used in filter hybridization with [^{32}P]poly(A)$^-$ RNA, hybridization to M13-IV(−) DNA was ~10 times higher than to M13-IV(+) DNA, suggesting that it mainly contains positive polarity RNA. To further substantiate this suggestion the influenza virus RNA specific for segments 4 (encoding haemagglutinin) and 5 (encoding nucleoprotein) were isolated by hybridization to 3′-terminal HA and NP

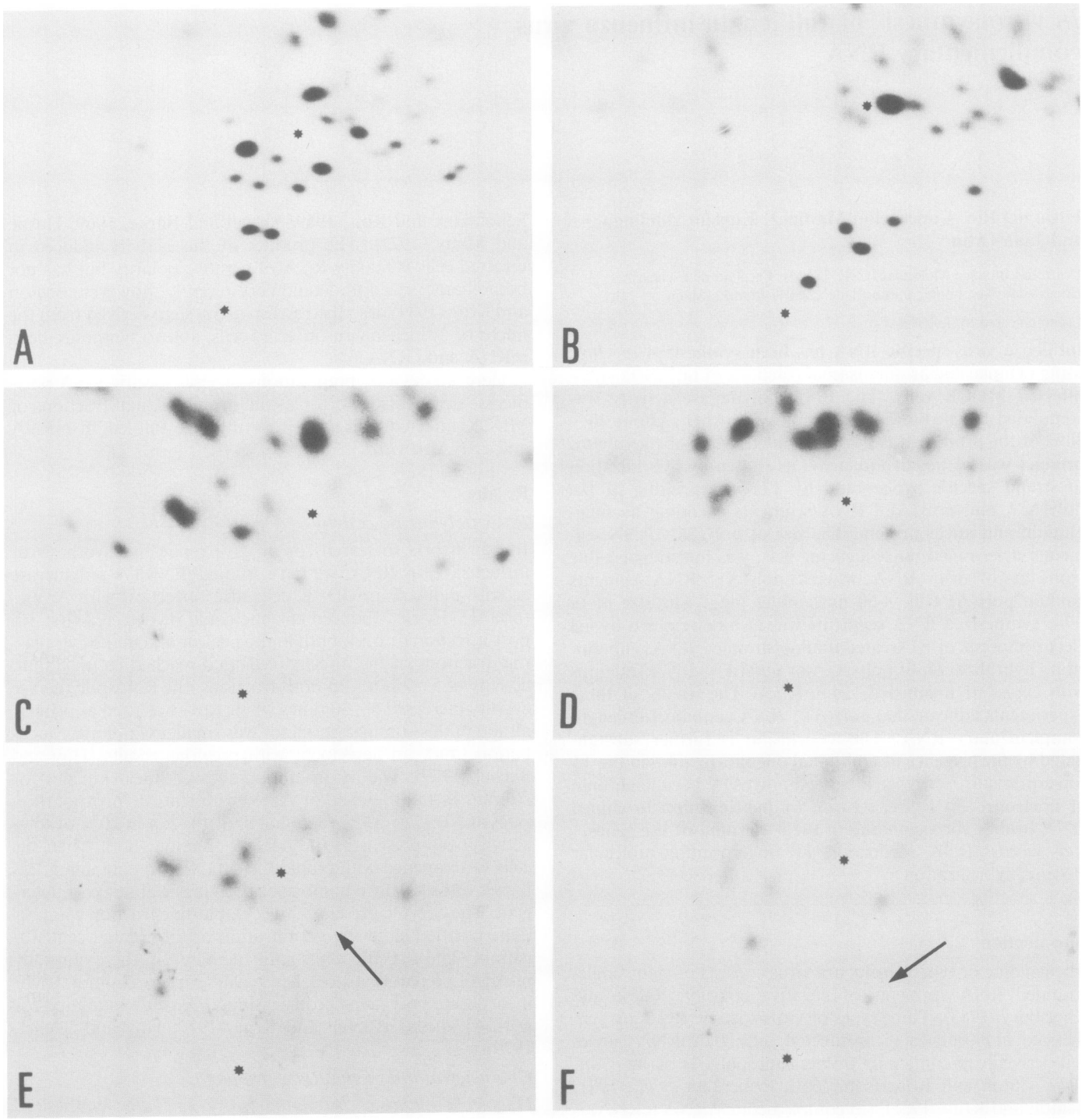

Fig. 1. T1 fingerprint analysis of *in vitro* synthesized RNA. *In vitro* synthesized RNA was fractionated through oligo(dT)-cellulose, hybridized to filters containing pHA3′ or pNP3′ DNA as described in Materials and methods and finally analyzed by T1 fingerprinting. **A, C** and **E**, NP-specific RNA; **B, D** and **F**, HA-specific RNA; **A** and **B**, *in vivo* labelled vRNA; **C** and **D**, poly(A)$^+$ RNA labelled *in vitro* with [α-^{32}P]UTP; **E** and **F**, poly(A)$^-$ RNA labelled *in vitro* with [α-^{32}P]UTP. Stars indicate the location of BPB and XC dye markers, and arrows show the position of additional T1 oligonucleotides in **E** and **F**, as compared with **C** and **D**.

DNA probes, and analyzed by T1 fingerprinting as described (Ortín *et al.*, 1980). The results of the T1 fingerprinting of the RNAs isolated from poly(A)$^+$ and poly(A)$^-$ RNA fractions are shown in Figure 1, together with the T1 fingerprints of *in vivo* labelled vRNA. The oligonucleotide composition of the hybrids indicates that the *in vitro* product essentially contains RNA of positive polarity. However, since the selection of RNA has been made by filter hybridization, a small proportion of synthesized RNA of negative polarity, if present, could have been lost by hybridization in the liquid phase to the excess of positive polarity RNA product.

The T1 fingerprints of both RNA segments 4 and 5 isolated from the poly(A)$^-$ RNA fraction (Figure 1E and F) show one additional large T1 oligonucleotide not present in the corresponding T1 fingerprints of the poly(A)$^+$ RNAs (Figure 1C and D). The size of these T1 oligonucleotides is that expected

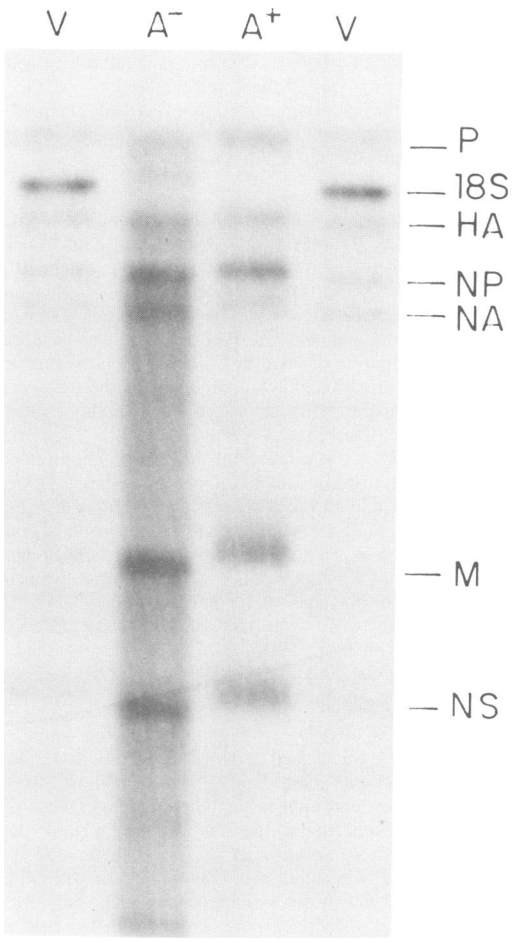

Fig. 2. Gel electrophoresis of *in vitro* synthesized RNA. Poly(A)$^-$ and poly(A)$^+$ RNA fractions of *in vitro* synthesized RNA were run in a 3% polyacrylamide-urea gel, together with *in vivo* labelled viron RNA as described in Materials and methods. V: virion RNA; A$^-$: poly(A)$^-$ RNA; A$^+$: poly(A)$^+$ RNA. Letters on the right margin indicate the position of virion RNA segments encoding the different viral genes, as well as 18S RNA, a frequent contaminant of virion preparations.

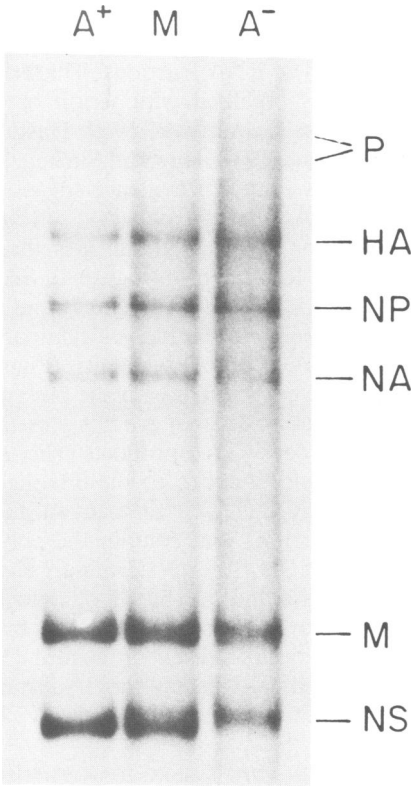

Fig. 3. Gel electrophoresis of double-stranded RNAs. Hybrids of poly(A)$^-$ or poly(A)$^+$ [^{32}P]RNA fractions of *in vitro* synthesized RNA with excess unlabelled virion RNA were treated with S1 nuclease and run on a 4% polyacrylamide gel as described in Materials and methods. A$^+$: poly(A)$^+$ RNA-virion RNA hybrid; A$^-$: poly(A)$^-$ RNA-virion RNA hybrid; M: mixture of the samples of A$^+$ and A$^-$.

from the sequence of the polyadenylation site of segments 4 and 5 (see below), suggesting that the poly(A)$^-$ RNA fraction contains full-length complementary RNA.

Size of the in vitro product. The results of hybridization to 3′-terminal DNA probes and T1 fingerprinting of selected RNAs, suggest that both poly(A)$^+$ and poly(A)$^-$ *in vitro* RNA fractions contain long, probably complete, RNA segments. Their size was determined in polyacrylamide-urea sequencing gels, using *in vivo* labelled vRNAs as markers. As can be seen in Figure 2, the poly(A)$^+$ RNA fraction shows a standard set of virus-specific RNA segments, except that they are 20–30 nucleotides longer than vRNA. Since mRNAs contain a sequence 17–22 nucleotides shorter than vRNA (Hay *et al.*, 1977; Plotch and Krug, 1978), it follows that *in vitro* synthesized mRNAs contain poly(A) tails of an average length of 50 nucleotides. The poly(A)$^-$ RNA fraction contains a higher level of fragmented RNA, but yet shows a distinct pattern of bands with mobilities almost indistinguishable from those of vRNA markers, again suggesting that, at least in part, the poly(A)$^-$ RNA fraction comprises full-length cRNA. Similar results were obtained when nuclear extracts were used as a source of enzyme (data not shown).

In spite of the use of sequencing gels, it is possible that the electrophoretic mobility does not exactly reflect the size of the RNAs. To avoid the possible complications due to secondary structure, poly(A)$^+$ and poly(A)$^-$ [^{32}P]RNA fractions were hybridized with excess unlabelled vRNA, the hybrids were treated with S1 nuclease and analyzed by polyacrylamide gel electrophoresis. Figure 3 shows that the mobility of the poly(A)$^+$ RNA-vRNA hybrid is slightly higher than that of the poly(A)$^-$ RNA-vRNA one. This mobility difference, which would account for a difference in size of 10–20 nucleotides, is apparent for segments 7 and 8. This result is compatible with the difference in size between mRNA and cRNA and agrees with similar data obtained for *in vivo* labelled RNAs (Hay *et al.*, 1977; Plotch and Krug, 1978).

Discussion

Our knowledge of the influenza virus transcription has improved recently, and a molecular model has been proposed for the role of the P proteins in the initiation and elongation of mRNA synthesis (Braam *et al.*, 1983). However, no similar progress has been made on the process of cRNA and vRNA synthesis. We have therefore attempted to set up an *in vitro* system able to support some type of influenza virus replicative RNA synthesis. The results presented above indicate that cytoplasmic or microsomal fractions of MDCK cells infected by influenza virus give rise to an *in vitro* RNA product that, at least in part, consists of full-length cRNA.

Only 20–30% of the *in vitro* RNA product was retained by oligo(dT)-cellulose columns, and contained poly(A) tails ~50 nucleotides long (Figure 2). The poly(A)$^-$ *in vitro* prod-

uct is mostly of positive polarity, as shown by T1 fingerprinting and by hybridization to strand-specific probes of both poly(A)$^+$ and poly(A)$^-$ RNA fractions. This is in agreement with previous results obtained with virion or infected cell associated polymerase (Scholtissek, 1969; Hastie and Mahy, 1973; Plotch and Krug, 1977; Hay and Skehel, 1979; Beaton and Krug, 1984). Two lines of evidence suggest that it contains full-length cRNA. First, the poly(A)$^-$-vRNA hybrid has a smaller mobility on polyacrylamide gels than the corresponding poly(A)$^+$-vRNA hybrid (Figure 3). Second, the T1 fingerprints of poly(A)$^-$ RNA segments 4 and 5 contain at least one extra T1 oligonucleotide, as compared with the corresponding poly(A)$^+$ RNA segments (Figure 1). Both types of evidence are compatible with the presence in cRNA of 16 3′-terminal nucleotides that are not present in mRNA, as previously shown for *in vivo* products (Hay *et al.*, 1977). These sequences would give rise to the T1 oligonucleotides UAAUUAAAAACACCCUUG (segment 4) and AAAAAUACCCUUG (segment 5) that contain the polyadenylation site, and UUUCUACU, the 3′-terminal oligonucleotide common to segments 4 and 5. The T1 oligonucleotides that contain the polyadenylation site correspond by size to the additional oligonucleotides present in Figure 1E and F, as compared with C and D, whereas the 3′-terminal oligonucleotide, if present, cannot be resolved in the T1 fingerprint shown.

The ability of the *in vitro* system to synthesize full-length cRNA could be related to the presence in the enzyme preparation of excess nucleoprotein or a modified form of nucleoprotein molecule, as compared with purified virions, but the influence of other viral proteins cannot be ruled out at this time. Recently, an anti-termination factor has been found in the cytoplasm of influenza virus-infected cells that is able to partially switch the synthesis of mRNA to cRNA (Beaton and Krug, 1984). The analysis of the activity of the enzyme derived from viral *ts* mutants, as well as the purification of the system, will provide clues to the mechanisms of cRNA termination.

Materials and methods

Viruses and cells

The A/Victoria/3/75 (H3N2) strain of influenza virus was used for all experiments. MDCK cells were obtained from The American Type Culture Collection. Molecular cloning was performed with the following host-vector systems: *Escherichia coli* HB101 (Boyer and Roulland-Dussoix, 1969) or *E. coli* MC1061 (Casadaban and Cohen, 1980) and pBR322 (Bolivar *et al.*, 1977) or *E. coli* JM101 and M13mp7 (Messing *et al.*, 1981), using described procedures (Maniatis *et al.*, 1982; Ortín *et al.*, 1983; de la Torre *et al.*, 1984).

Enzymes and chemicals

Restriction endonucleases, *E. coli* DNA pol I (Klenow fragment), T4 DNA ligase and polynucleotide kinase were from New England Biolabs, and alkaline phosphatase from Boehringer. RNase T1 was bought from Calbiochem, S1 nuclease from Sigma Chemical Co. and reverse transcriptase was a gift from J.Beard (Life Sciences Inc.). Radioactive triphosphates ([α-^{32}P]GTP, [α-^{32}P]UTP and [^{3}H]UTP), [^{32}P]phosphate and RNasin were obtained from The Radiochemical Centre (Amersham). Unlabelled triphosphates were obtained from Sigma and filtered through Sephadex G10 columns to remove traces of contaminant RNases. Dinucleotide ApG, oligo(dT)-cellulose and dithiothreitol (DTT) were from Sigma Chemical Co. All other chemicals were reagent grade.

Extract preparation

Infected MDCK cells were lysed and fractionated as described previously (Ortín and Doerfler, 1975) except that lysis buffer was 0.5% NP-40 in HB buffer (10 mM KCl, 1.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 10 mM Tris-HCl, pH 8.5). In some experiments, cells were opened in the same buffer lacking NP-40, by using a Dounce homogenizer. Nuclear pellets were resuspended in 44% glycerol in HB buffer and stored at −80°C. To obtain the cytoplasmic fraction, the supernatant was centrifuged for 10 min at 30 000 *g* and 0°C. Virus-specific RNPs were purified from the cytoplasmic fraction by filtration at 4°C through a Sephadex G200 column equilibrated with 2 x HB buffer. Enzymatic activity was located in the fractions of the excluded volume. Alternatively, the cytoplasmic fraction was centrifuged for 1 h at 0°C and 100 000 *g* and the microsomal pellet was resuspended in 44% glycerol in HB buffer and stored at −80°C.

In vitro synthesis of RNA

Standard reaction mixtures contained up to 15 μg of protein in 20 μl of 50 mM Tris-HCl pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 U/μl RNasin, 2 mM unlabelled triphosphates and either 100 μM [α-^{32}P]UTP or 50 μM[α-^{32}P]GTP. When present, ApG was at 40−80 μM. When nuclear extracts were used, actinomycin D was present at 20 μg/ml. Unless otherwise stated, reaction was for 60 min at 30°C. Incorporation was measured by trichloroacetic acid precipitation of aliquots of the reaction mixture and determination of Cerenkov radiation. Each component of the reaction mixture, including the enzyme preparation, was tested for its ability to degrade Qβ RNA, and shown to be RNase free.

In vitro synthesized RNA was extracted from reaction mixtures as follows. The reaction was stopped by adjusting to 0.1 M NaCl-50 mM Tris 7.5-5 mM EDTA and 0.5% SDS and the mixture was incubated with proteinase K (100 μg/ml) for 10 min at 37°C. After three extractions with phenol-chloroform-isoamylalcohol-hydroxyquinoline mixture (Holmes and Bonner, 1973), the RNA was excluded of Sephadex G50 in a spun column (Maniatis *et al.*, 1982) equilibrated with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE buffer). Genomic RNA was extracted from purified labelled or unlabelled virions as previously described (Ortín *et al.*, 1980).

Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as standard.

Olig(dT)-cellulose chromatography

In vitro synthesized RNA was heated at 100°C for 1 min in TE buffer, immediately diluted 10 times with 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.5% SDS at room temperature and applied to the oligo(dT)-cellulose column at ~0.5 ml/min. After the collection of the flow-through [poly(A)$^-$ RNA], the column was washed with 10 volumes of the same buffer and the bound material [poly(A)$^+$ RNA] was eluted with five volumes of 10 mM Tris-HCl, pH 7.5, 0.5% SDS and then with five volumes of H_2O. The proper separation of the fractions was checked by re-chromatography. Finally, both poly(A)$^-$ and poly(A)$^+$ RNA were recovered by ethanol precipitation.

Preparation of DNA probes

Random cloning into M13mp7. To prepare strand-specific probes, viral DNA was synthesized *in vitro* as described (Ortín *et al.*, 1983) and digested with *Sau*3AI endonuclease. Digested DNA (10 ng) was ligated to 50 ng of *Bam*HI digested, phosphatase-treated M13mp7RF DNA and the product was used to transform competent *E. coli* JM101 cells. To determine whether vRNA or cRNA sequences were represented in the virus-specific inserts, two radioactive probes were prepared: vRNA was labelled with [γ-^{32}P]ATP and polynucleotide kinase after partial digestion with T1 ribonuclease, and a cDNA copy was synthesized including [α-^{32}P]dATP as precursor. The screening of recombinant M13mp7 plaques with these probes by *in situ* hybridization (Benton and Davis, 1977), yielded two collections of clones, one with positive polarity inserts [M13-IV-DNA(+)] and the other with negative polarity inserts [M13-IV-DNA(−)].

Preparation of 3′-terminal HA and NP DNA probes. Plasmid pPR5, that contains the complete sequence of A/PR/8/34 NP gene cloned into the pBR322 *Pst*I site by dG:dC tailing (Ortín, unpublished results), was digested with *Pvu*I endonuclease, ligated at a concentration of 1 μg/ml and used to transform competent *E. coli* MC1061 cells. The resulting clone, pNP 3′, was shown by restriction analysis to contain the 3′-terminal 95 nucleotides of RNA segment 5. Clone pHA 3′ was prepared by digestion with *Bam*HI endonuclease of plasmid 5_2/13, that contains the 3′-terminal third of the A/Bangkok/1/79 HA gene cloned in the *Hind*III site of pBR322 (Martínez and Ortín, unpublished results), and ligation as described above. It contains the 3′-terminal 150 nucleotides from the *Bam*HI site through the 3′ terminus of HA gene.

Molecular hybridization

Hybridization of *in vitro* synthesized RNA to DNA immobilized on filters was performed as described (Ortín *et al.*, 1980) except that the hybridization buffer did not contain formamide and the hybridization temperature was 66°C. For liquid hybridization, *in vitro*-labelled RNA and excess unlabelled virion RNA were ethanol precipitated in the presence of 10 μg yeast RNA as carrier. The precipitate was resuspended in 10 μl of TE, boiled for 1 min, cooled in ice and mixed with 10 μl of 1.0 M NaCl, 40 mM Tris-HCl, pH 7.5, 2 mM EDTA. Hybridization was for 3 h at 60°C. The mixture was cooled in ice, diluted 10 times with 0.28 M NaCl-40 mM Na acetate pH 4.6, 1 mM

$ZnSO_4$ and incubated 30 min at 37°C with 10 U of S1 nuclease. Finally, the hybrids were recovered by ethanol precipitation with 10 μg of yeast RNA as carrier.

Gel electrophoresis

For size determination, RNA in 3 μl of 0.5 mM EDTA was heated at 100°C for 1 min, mixed with 2 μl of xylene cyanol (XC) and bromophenol blue (BPB) dye markers in 50% formamide, saturated with solid urea and loaded onto 3% polyacrylamide, 0.6 mm thick sequencing gels (Maxam and Gilbert, 1980). Electrophoresis was at 45 W (25 V/cm) until XC dye marker ran off the gel. Double-stranded RNA was run in 4% polyacrylamide gels in TBE buffer (Peacock and Dingman, 1967) for 20 h at 5 V/cm.

T1 fingerprinting (de Wachter and Fiers, 1972) was as described (Domingo *et al.*, 1980; Ortín *et al.*, 1980). Gels were dried and exposed at −70°C with intensifying screens.

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