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REPLICATION

Structure and synthesis of influenza virus complementary RNAs

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Two classes of genome transcript are involved in the replication of influenza viruses, both of which contain molecules complementary to all eight genome RNAs. One of these classes, which comprises the viral mRNAs, consists of transcripts that have additional 5′ terminal non-viral sequences including 'cap' structures and 3′ terminal polyadenylate sequences but which lack sequences corresponding to nucleotides 1–16 at the 5′ termini of the virus RNAs. The members of the other class are complete genome transcripts with no additional sequences and these are thought to be the templates for genome replication. The syntheses of the two classes of molecules have a number of distinctive features which together with their structural properties indicate that they are synthesized by different mechanisms.

Introduction

During influenza virus replication, transcripts of the virus genome have two functions, as messenger RNAs and as template for replicating the genome; as in the other 'negative-strand' viruses such as rhabdoviruses and paramyxoviruses these roles appear to be carried out by distinct types of transcript. Two classes of complementary RNA are synthesized during influenza infection (Hay *et al.* 1977*a*). The components of one of these are mRNAs whereas the component of the other class have several characteristics consistent with a role in genome replication and this paper considers the primary structure and synthesis of these molecules in relation to their functions.

The composition of complementary RNA (cRNA) in virus-infected cells has been examined by annealing radioactively labelled cRNAs to unlabelled virion RNA and analysing the double-stranded molecules by polyacrylamide gel electrophoresis. The results of such analyses, as illustrated in figure 1, showed that the two classes of cRNA, which can be separated by oligo-(dT)-cellulose chromatography since only one of them has additional 3′ poly(A) sequences, contain transcripts of all eight genome RNAs. The observation that in infected cells only the polyadenylated molecules are associated with polysomes (Hay *et al.* 1977*a*) strongly suggests that, although in cell-free protein synthesizing systems both polyadenylated and non-polyadenylated transcripts are translated (Stephenson *et al.* 1977), *in vivo* this function is reserved for the former molecules.

Synthesis of complementary RNAs

Two phases of transcription can be distinguished during virus infection. The first of these, primary transcription, which occurs independently of protein synthesis and involves transcription of the genome of infecting virus by the virion-associated RNA polymerase, results in the synthesis of only polyadenylated cRNAs. Polypeptides translated from these mRNAs are

required for secondary transcription, which appears to involve initially the synthesis of a second class of cRNA which is used to synthesize progeny viral RNA which in turn can be transcribed into mRNAs. With respect to these general features of RNA synthesis the continual dependence on protein synthesis of the production of both viral RNA and non-polyadenylated cRNA (Scholtissek & Rott 1970; Hay *et al.* 1978) is consistent with the proposed function of the latter in genome replication.

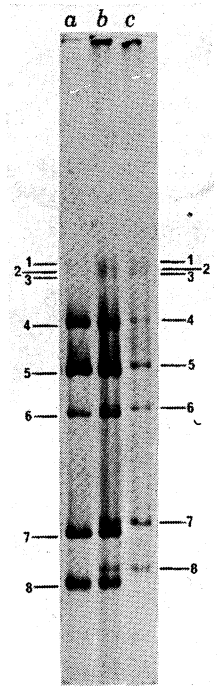

FIGURE 1. Analysis of double-stranded RNAs formed between virus RNA and ^{3}H-polyadenylated or non-polydenylated cRNAs. RNA extracted from chick cells infected with fowl plague virus and incubated with [^{3}H]uridine was fractionated by oligo(dT)-cellulose chromatography, hybridized with excess unlabelled virus RNA and digested with nuclease S_1 and the double-stranded RNAs analysed by polyacrylamide gel electrophoresis and fluorography as described by Hay *et al.* (1977*a*). The double-stranded RNAs from polyadenylated (*a*) and non-polyadenylated (*c*) hybrids were analysed separately or as a mixture (*b*).

The syntheses of the two classes of cRNA in fowl plague virus infected chick cells exhibit a number of distinctive features (Hay *et al.* 1977*a*). Whereas both mRNA and viral RNA are synthesized at maximum rate approximately 2 h after infection, the synthesis of non-polyadenylated cRNAs reaches a maximum some 45 min earlier. The amounts of the two classes of cRNA produced at these times are also quite different, the maximum rate of mRNA production being approximately tenfold that of the non-polyadenylated cRNAs. In addition, their compositions are quite characteristic and, as shown in table 1, whereas at 3 h after infection the relative abundance of the different non-polyadenylated cRNAs varies by no more than twofold, the disparity in the amounts of the different mRNAs is much greater. With regard to the function of these molecules it is notable that there is a close correlation between the relative abundance of the different mRNAs and the relative rates of synthesis of the corresponding polypeptides; it is apparent from comparisons at different times during infection and between different cells infected by the same virus (Bosch *et al.* 1978) that the rate of polypeptide synthesis is predominantly determined by the amount of the particular mRNA.

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The differences between the syntheses of the two classes of cRNA are further emphasized by analyses at different times during infection. Within 30 min of infection both are synthesized in similar relative amounts; however, whereas the relative rates of production of the various non-polyadenylated cRNAs remain relatively unaltered over the next 2 h, the pattern of mRNA synthesis varies quite markedly. For example, between 30 and 90 min after infection mRNAs 5 and 8 are preferentially synthesized while at later times mRNAs 4, 5 and 7 are synthesized in greatest amount. mRNA 1, 2 and 3, on the other hand, are synthesized in relatively small amounts throughout infection. Thus, in addition to the disparity in the amounts of the various mRNAs produced, the times at which they are synthesized at maximum rate differ. In contrast, although at later times there is a decrease in the relative amount of cRNA 8 produced, the non-polyadenylated cRNAs all attain their maximum rate of synthesis at the same time.

TABLE 1. COMPARISONS OF THE COMPOSITIONS OF POLYADENYLATED AND NON-POLYADENYLATED cRNAs AND THE RELATIVE RATES OF SYNTHESIS OF VIRUS-SPECIFIC POLYPEPTIDES

	relative amounts of cRNA		relative rates of synthesis of polypeptides	
RNA species	−poly(A)	+poly(A)		
1	0.70	0.06	P_1	0.04
2	0.82	0.09	P_2	0.04
3	0.62	0.07	P_3	0.03
4	0.70	0.30	HA	0.41
5	1.00	1.00	NP	1.00
6	0.88	0.24	NA	—
7	0.90	0.36	MP	0.33
8	0.50	0.36	NS	0.35

The data on cRNA composition were obtained from analyses of chick cells infected with fowl plague virus and incubated for 3 h in medium containing [^{32}P]orthophosphate, as described by Hay *et al.* (1977*a*). The relative rates of synthesis of virus-specific polypeptides were estimated from analyses of extracts of cells labelled for 10 min with ^{14}C-protein hydrolysate at 3 h after infection as described by Bosch *et al.* (1978). The numerical data are expressed relative to RNA 5 or nucleoprotein polypeptide.

Further evidence that the syntheses of the two classes of cRNA are regulated differently comes from comparative investigations of transcription in L-cells infected with fowl plague virus. Whereas the syntheses of the non-polyadenylated cRNAs are similar in the two systems, the patterns of mRNA synthesis are quite distinct and in particular the synthesis of mRNAs 6 and 7 in L-cells is depressed (Bosch *et al.* 1978). A peculiar feature of transcription in cells infected with a *ts* mutant of fowl plague virus, *ts* 166 (Ghendon *et al.* 1975), shows that, in some cases at least, the relative rates of synthesis of the different non-polyadenylated cRNAs can vary dramatically during infection. Whereas early in infection, synthesis is similar to that in wild-type virus-infected cells, after 1½ h there is a predominant synthesis of non-polyadenylated cRNA 6, although there is no corresponding increase in synthesis of mRNA 6.

The syntheses of the two classes of cRNA are further distinguished by their sensitivities to inhibitors of protein and host cell DNA-dependent RNA syntheses. Whereas mRNA synthesis continues in cells infected in the presence of cycloheximide (primary transcription) or after addition of the drug during infection, the synthesis of non-polyadenylated cRNA is inhibited. This dependence of non-polyadenylated cRNA synthesis on continued protein synthesis is reflected in the phenotypes of several *ts* mutants of fowl plague virus. Mutants in four different complementation groups were found to be defective in the synthesis of non-polyadenylated cRNA at

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non-permissive temperature while only one of these showed a defect in mRNA production in 'shift-up' experiments (A. J. Hay and Y. Ghendon, unpublished results). It therefore appears that the continued synthesis of several virus polypeptides, not involved in mRNA synthesis, is required for non-polyadenylated cRNA production and that different enzymes are responsible for the synthesis of the two classes of cRNA. There is as yet little information concerning the polypeptide composition of these enzymes, although analyses of the genetic defects of *ts* mutants of different influenza viruses have suggested that the product of gene 1 is involved in both mRNA and non-polyadenylated cRNA synthesis and that the products of genes 2, 3 and 5 are required for the synthesis of non-polyadenylated cRNA and probably also of vRNA (Y. Ghendon, personal communication; Palese 1977; Scholtissek 1978; Barry & Mahy 1979). Addition of actinomycin D during influenza infection depresses the synthesis of viral mRNA to an extent similar to that of host cell RNA synthesis whereas non-polyadenylated cRNA synthesis is affected to a much lesser degree and as discussed later it appears that this distinction reflects differences in the mechanisms of initiation of synthesis of the two types of molecules.

TABLE 2. 5′ TERMINAL NUCLEOTIDE SEQUENCES OF RNAs OF FOWL PLAGUE, X-31 AND B/HONG KONG/8/73 INFLUENZA VIRUSES

	1 5 10 15 20 23
FPV 1–3	AGUAGAAACAAGGUAGUUUUUUA
4	AGUAGAAACAAGGGAGUUUUUUU
5	AGUAGAAACAAGGGUAUUUUUUU
6	AGUAGAAACAAGGAGAUUUUUUU
7	AGUAGAAACAAGGUAGUUUUUUA
8	AGUAGAAACAAGGGUGUUUUUUA
9	AGUAGAAACAAGGUAGUUUUUUA
10	AGUAGAAACAAGGUAGUUUUUUA
X-31 1–3	AGUAGAAACAAGGUAGUUUUUUA
4	AGUAGAAACAAGGGUGUUUUUUA
5+6	AGUAGAAACAAGG(G/A)(U/G)AUUUUUUA
7	AGUAGAAACAAGGUAGUUUUUUA
8	AGUAGAAACAAGGGUGUUUUUUA
B/HK 1	AGUAGAAACACGAGCAUUUUUAA
2	AGUAGAAACAAGAGCAUUUUUUA
3	AGUAGAAACAAGAGCAUUUUUUA
4	AGUAGAAACAAG(A/G)GAAUUUUUUA
5	AGUAGAAACAAGAGGAUUUUUAA

PRIMARY STRUCTURE

Analyses of the nucleotide sequences of the two types of cRNA have indicated that in addition to the presence or absence of 3′ terminal poly(A) sequences (Plotch & Krug 1977) there are other differences in their primary structure. Differences in the electrophoretic mobilities of the double-stranded molecules formed between virus RNA and the corresponding cRNAs of the two classes, as shown in figure 1, initially suggested that the polyadenylated transcripts are shorter. This has since been confirmed by analyses of the susceptibility to nuclease S_1 digestion of the 5′ and 3′ terminal nucleotides of virus RNAs in hybrids with the two types of cRNA, by ribonuclease T_1 oligonucleotide fingerprint analyses of either cRNA or viral RNA in the double-stranded regions of hybrid molecules, and by indirect nucleotide sequence analyses. Both the

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5′ and 3′ terminal nucleotides of virus RNAs in hybrids with non-polyadenylated cRNAs are protected against nuclease S_1 digestion, whereas only the 3′ termini of viral RNAs are protected by mRNAs (Hay *et al.* 1977*b*; Skehel *et al.* 1979). Nucleotide sequence analyses of the single-stranded 5′ terminal regions of the virus RNAs present in these hybrids have indicated that nucleotides 1–16 are not represented in the mRNAs, although the exact point of termination has yet to be established (Skehel & Hay 1978). It is of particular interest that apart from a variable triplet, residues 14–16, the sequences of the first 22 nucleotides at the 5′ ends of all

TABLE 3. COMPARISONS OF THE 5′ TERMINAL AND 3′ TERMINAL NUCLEOTIDE SEQUENCES OF FPV RNAs 4–8

		1 5 10 15
RNA 4	5′	AGUAGAAACAAGGGAG
	3′	UCGUUUUCGUCCCCU
RNA 5	5′	AGUAGAAACAAGGGUA
	3′	UCGUUUUCGUCCUAU
RNA 6	5′	AGUAGAAACAAGGAGA
	3′	UCGUUUUCGUCCUCU
RNA 7	5′	AGUAGAAACAAGGUAG
	3′	UCGUUUUCGUCCAUC
RNA 8	5′	AGUAGAAACAAGGGUG
	3′	UCGUUUUCGUCCCAC

RNAs of different influenza A viruses are identical and very similar to those of the RNAs of influenza B viruses (table 2) (see also Barry & Mahy 1979). The sequences of nucleotides 1–12 at the 3′ ends of the various genome RNAs of different influenza A viruses also appear identical (Skehel & Hay 1978; see table 4). Although there are clear differences between the conserved sequences at the 3′ ends of the genome RNAs and their complete transcripts, the apparent similarities, e.g. the relative absence of A residues, may reflect their functional relatedness since both are presumably involved in initiating RNA synthesis, the former in transcription and the latter in replication of the virus RNAs. A particularly notable feature of RNAs 6, 7 and 8 which may be related to these functions is the complementarity between the hexanucleotides at residues 11–16 of the 5′ end, which includes the variable triplet, and at residues 10–15 of the 3′ end of each of these RNAs (table 3). This does not, however, appear to be a reproducible phenomenon since in RNAs 4 and 5 the complementarity of the variable triplet is not complete. An additional point evident from these results is that there is no specific complementarity between the 5′ and 3′ termini of the different genome segments which might have provided a mechanism for the selection or ordered replication of the full genome complement.

Analyses of the 3′ terminal nucleotide sequences of fowl plague virus RNAs and the 5′ terminal sequences of their transcripts indicate that for transcripts 4–8 the first AUG triplet is located between residues 20 and 30 (table 4); however, it remains to be established whether or not these are the sites of initiation of polypeptide synthesis. Apart from the conserved sequence of nucleotides 1–12 no further similarities are apparent in these sequences of the various RNAs and there does not appear to be any structural basis for selective initiation of transcription of for example RNAs 5 and 8, the mRNAs of which are preferentially synthesized early during

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TABLE 4. 5′ TERMINAL NUCLEOTIDE SEQUENCES OF IN-VITRO TRANSCRIPTS OF FPV RNAs

	1 5 10	15 20 25 30
transcript 4	AGCAAAAGCAGG	GGAUAAAAAAUGAAC
5	AGCAAAAGCAGG	AUAGAUAAAUGACA
6	AGCAAAAGCAGG	AGAACAAAAUGAAU
7	AGCAAAAGCAGG	UAGAUAUUGAAAGAUGA
8	AGCAAAAGCAGG	GUGACAAAAACAUAAUGG

secondary transcription. An interesting feature which has emerged from comparisons of the 5′ terminal nucleotide sequences of the in-vitro transcripts of RNAs 4–8 of fowl plague virus, X-31 (H3N2) and A/Japan/57 (H2N2) is that whereas the equivalent sequences of RNAs 5, 6, 7 and 8 of the different viruses show a high degree of similarity, there are marked differences in the sequences of transcript 4, in particular the first AUG triplet in the case of X-31 is at

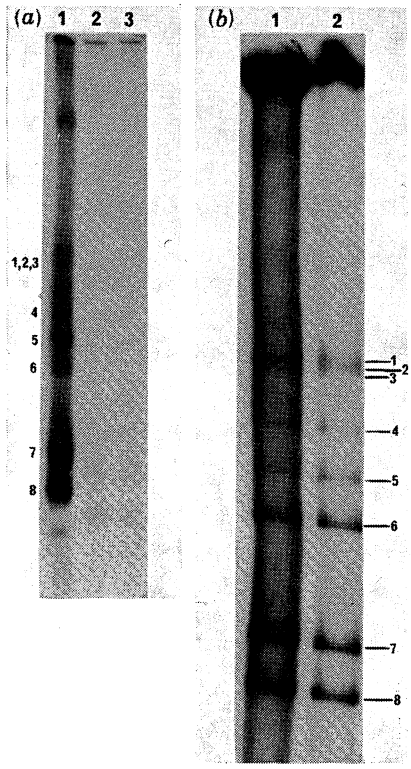

FIGURE 2. Nuclease susceptibility of the 5′ termini of polyadenylated (*a*) and non-polyadenylated (*b*) cRNAs in hybrids with virus RNA. Hybrid molecules containing virus RNA and either polyadenylated or non-polyadenylated cRNA were isolated from the RNA of fowl plague virus infected chick cells by a combination of chromatography on oligo(dT)-cellulose and CF-11 cellulose (Franklin 1966). The 5′ terminal 'cap' of the polyadenylated cRNAs were removed by periodate oxidation and β-elimination as described in Ziff & Evans (1978). The 'de-capped' polyadenylated and non-polyadenylated hybrids were treated with bacterial alkaline phosphatase and end-labelled by using polynucleotide kinase and [γ-^{32}P]ATP as described in Skehel & Hay (1978). The labelled vRNAs were removed by two cycles of hybridization with excess unlabelled virus RNA followed by chromatography on either oligo(dT)-cellulose (polyadenylated hybrids) or CF-11 cellulose (non-polyadenylated hybrids). Equal aliquots dissolved in 0.3 M NaCl, 1 mM $ZnSO_4$, 10 mM sodium acetate, pH 4.5, were analysed before or after incubating at 37 °C with nuclease S_1 (20 units/μg RNA). (*a*) Hybrids containing 5′ terminally labelled polyadenylated cRNAs either untreated (1) or treated with nuclease S_1 for 10 min (2) or 60 min (3). (*b*) Double-stranded RNAs containing 5′ terminally labelled non-polyadenylated cRNAs either untreated (1) or treated with nuclease S_1 for 30 min (2).

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nucleotides 30–32 and at around nucleotide 45 in the A/Japan/57 transcript. In addition, analyses of the nucleotide sequences near the 5′ termini of the corresponding RNAs of these viruses have shown that for RNAs 7 and 8 there is a high degree of similarity whereas the equivalent sequences of RNA 4 are quite different. This clearly reflects the greater variation in the haemagglutinin gene and also indicates that gene 4 of the two human viruses in particular are not related by limited mutation, which is consistent with conclusions derived from amino acid sequence analyses of haemagglutinins of different subtypes (see, for example, Laver 1973) and RNA–RNA hybridization analyses (Scholtissek *et al.* 1977).

Analyses of the nucleotide sequences at the 5′ termini of cRNAs synthesized *in vivo* have shown that the non-polyadenylated cRNAs do not possess 5′ terminal 'cap' structures and that the terminal dodecanucleotide sequences are exact complements of those at the 3′ ends of the genome RNAs. The mRNAs, on the other hand, in addition to the 7-methylguanosine in 'cap' structures (Krug *et al.* 1976) appear to possess additional non-viral sequences at their 5′ ends. Figure 2*a*, *b* shows that whereas the 5′ terminal nucleotides of non-polyadenylated cRNAs in double-stranded molecules are resistant to nuclease S_1 digestion, most of the 5′ termini of mRNAs in hybrids with virus RNA are susceptible. Preliminary analyses suggest that the additional sequences are heterogeneous but similar for the different mRNAs, and analyses of 5′ terminal ribonuclease T_1 oligonucleotides indicate that some of these are at least 15 nucleotides in length.

Discussion

In summary, therefore, the two classes of cRNA differ in primary structure at both their 5′ and 3′ ends (table 5). Whether this is due to differences in initiation and termination of their synthesis or post-transcriptional modification remains to be established. With regard to the synthesis of mRNAs, the sensitivity of RNA transcription to inhibitors of DNA transcription such as actinomycin D and α-amanitin (Rott & Scholtissek 1970; Scholtissek & Rott, 1970; Pons 1977) and its insensitivity to α-amanitin in virus-infected α-amanitin-resistant cells (Lamb & Choppin 1977; Spooner & Barry 1977) suggested that RNA products of the host cell polymerase II are required for transcription. The demonstration that *in vitro* cell mRNAs can prime transcription by the virion-associated RNA polymerase reinforces this view and it has been suggested that sequences of these primers including the 5′ terminal 'cap' structure are incorporated into the transcript (Bouloy *et al.* 1978). It therefore appears likely that a similar mechanism accounts for the *in-vivo* incorporation into viral mRNAs of additional 5′ terminal non-viral sequences. The apparent heterogeneity of these sequences suggests that no single RNA polymerase II product is involved but rather a spectrum of products such as the cell mRNAs or their precursors. With regard to the observed differences in mRNA synthesis in different cells infected by the same virus, this may reflect variations in the RNA primers available. The possibility that non-polyadenylated cRNAs are synthesized with additional 5′ terminal sequences that are subsequently removed cannot as yet be discounted; however, the relative insensitivity of their synthesis to actinomycin D is consistent with the initiation of their synthesis being independent of a similar primer requirement. Furthermore, the differences noted in synthesis of the two classes of cRNA are quite consistent with different mechanisms for their synthesis.

The production of incomplete polyadenylated transcripts could be the result either of nucleolytic processing of complete transcripts or of termination before transcription of the 5′

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TABLE 5. NUCLEOTIDE SEQUENCES OF THE CONSERVED REGIONS OF GENOME RNAs AND THEIR TRANSCRIPTS

		1 5 10	20 15 10 5 1	
vRNA	(3′)	HO-U-C-G-U-U-U-U-C-G-U-C-C————	————U-U-U-U-U-U-(- -)†-G-G-A-A-C-A-A-AG-A-U-G-Appp	(5′)
mRNA	(5′)	m^7G-----A-G-C-A-A-A-A-G-C-A-G-G-————	————---A-A-$A_{(\sim 150)}$-A-OH	(3′)
cRNA (−poly(A))	(5′)	‡(pp)pA-G-C-A-A-A-A-G-C-A-G-G————	————A-A-A-A-A-A-(- -)-C-C-U-U-G-U-U-U-C-U-A-C-U-OH	(3′)

† The sequence of nucleotides 14–16 at the 5′ ends of vRNA is variable.
‡ The degree of phosphorylation is not known.

terminal regions of the virus RNAs. Since *in vitro* or *in vivo* in the absence of protein synthesis, incomplete transcripts only are produced (Hay *et al.* 1978), the latter alternative is more likely and it is possible that the U_6 sequence of residues 17–22 at the 5′ ends of all virus RNAs has some role in this process.

Finally, although there is as yet no direct evidence of the function of the non-polyadenylated cRNAs, certain features of their synthesis as pointed out above, as well as their structural characteristics are certainly consistent with their involvement as template in genome replication, a role which the incomplete mRNA transcripts could not perform.

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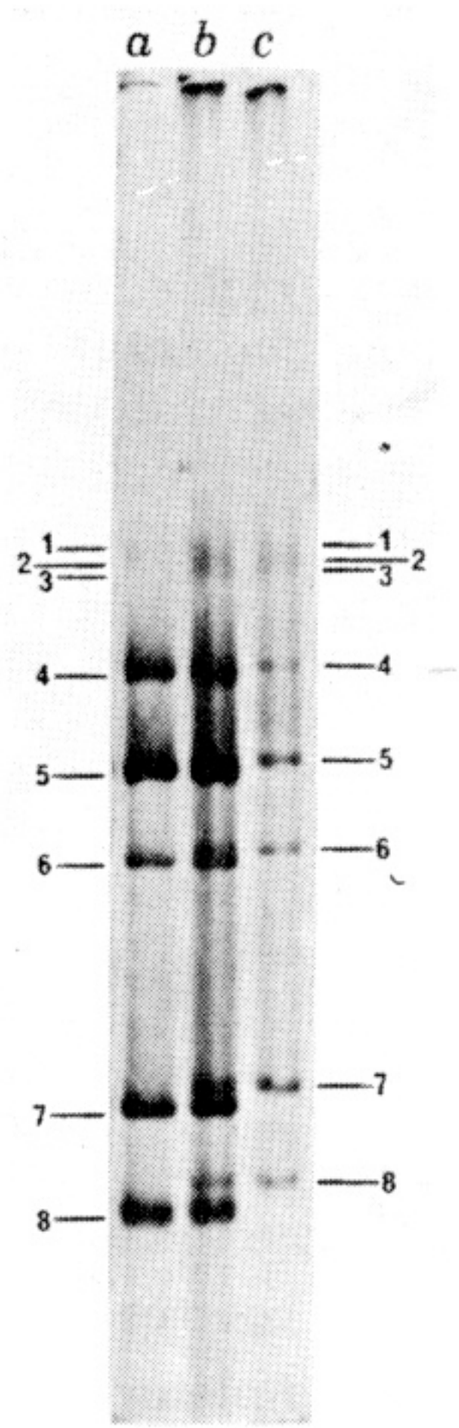
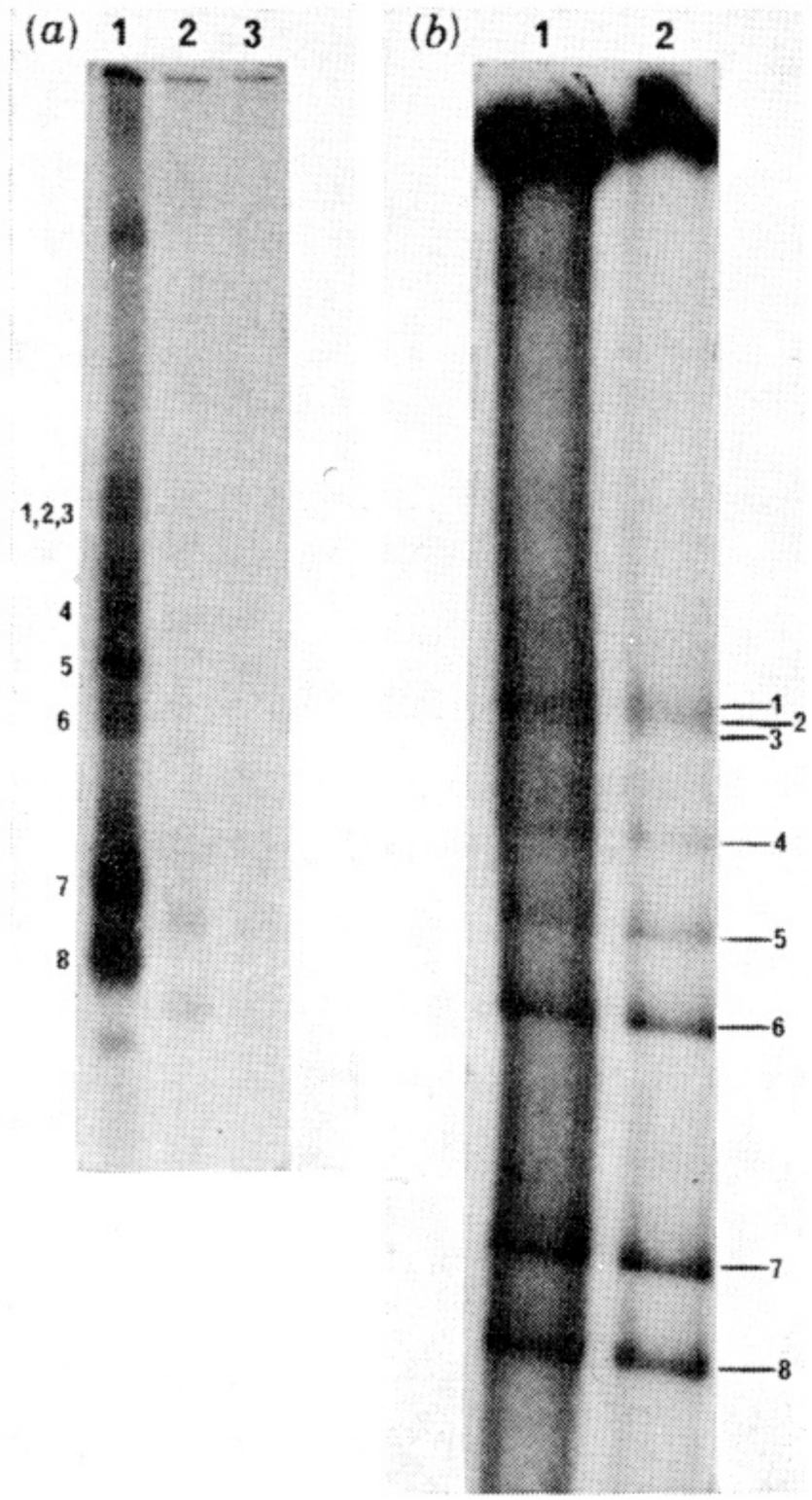


FIGURE 1. Analysis of double-stranded RNAs formed between virus RNA and ^{3}H-polyadenylated or non-polydenylated cRNAs. RNA extracted from chick cells infected with fowl plague virus and incubated with [^{3}H]uridine was fractionated by oligo(dT)-cellulose chromatography, hybridized with excess unlabelled virus RNA and digested with nuclease S_1 and the double-stranded RNAs analysed by polyacrylamide gel electrophoresis and fluorography as described by Hay *et al.* (1977*a*). The double-stranded RNAs from polyadenylated (*a*) and non-polyadenylated (*c*) hybrids were analysed separately or as a mixture (*b*).



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